Daphne Sudden Death Syndrome (DSDS):

Pathogen Identification, Characterization and Screening

For Disease Resistance

by

David Noshad

B.Sc. (Hons), University of Tabriz (Azerbaijan)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Plant Science)

THE UNIVERSITY OF BRITISH COLUMBIA

December 2007

ABSTRACT

Daphne is a widely dispersed genus with large variation in morphology, native habitats, and use. Unfortunately, broader acceptance of *Daphne* in the ornamental trade has been limited due to Daphne Sudden Death Syndrome (DSDS), a disease that kills the plant without warning. The results of this research identified *Thielaviopsis basicola* (Berk. et Br.) Ferr. as the causal agent for this disease. Pure cultures of the pathogen were developed and used in a germplasm screen.

To evaluate *Daphne* germplasm *in vitro*, species-specific protocols were developed that alleviated two common problems in *Daphne* micropropagation, browning and hyperhydricity. Optimizing the concentrations of both PGRs and charcoal was able to control these problems. Selected species were evaluated for resistance against *Thielavipsis basicola* in both, *in vivo* and *in vitro*, conditions. The results of both methods displayed a strong correlation and indicated significant differences among the taxa. However, there were differences in disease progression rates. Typically, the in vitro challenge produced a comparable level of disease as the in vivo challenge but in two to three weeks less time. Across both screening methods, the most resistant species evaluated were *D. tangutica* and *D. retusa,* while *D. cneroum* was the most susceptible.

Based on ITS sequences, phylogenetic relationships among selected *Daphne* species were established and associated with their resistance against *T. basicola*. The phylogeny indicated that *Daphne* is possibly a monophyletic group. However, placement of *D.*

genkwa remained problematic. The analysis of ITS sequences data resulted in a parsimony consensus tree with two well-supported major clades and one clade with less support. In general, the evolutionary tree for resistance, inferred from the phylogenetic data and the results of the screening project, indicate that resistance is a derived character and that plants recently evolved this ability.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abbreviations

BA: 6- benzyl aminopurine

DI: disease incidence

DS: disease severity

dsRNA: double stranded RNA

DSDS: Daphne Sudden Death Syndrome

IAA: indole-3 acetic acid

IBA: indole 3- butyric acid

NAA: α-naphthalene acetic acid

MS: Murashige and Skoog medium

PDI: plant disease index

PGR: plant growth regulator

TDZ: thidiazuron

WPM: woody plant medium

PREFACE

'To see a world in a grain of sand

And a heaven in a wild flower,

Hold infinity in the palm of your hand

And eternity in an hour.'

William Blake

ACKNOWLEDGEMENT

Many people contributed significantly to this project. My research supervisor and four committee members guided this study from its beginning to its final completion, and my gratitude for their valuable input and support is profound. I take this opportunity to thank Andrew Riseman for his great help. Without his help, this study could not have been accomplished. I also thank Zamir Punja, Murray Isman, Brian Ellis and Quentin Cronk for providing critical feedback and guidance on my project.

Many thanks to Vippen Jushi and her colleagues, at the BC Ministry of Agriculture, for helping in the pathogen identification; Douglas Justice, Ron Rollo, and their colleagues at the UBC Botanical Garden, for helping me with the plant material.

Finally, my deepest appreciation goes to my family and friends. Special thanks to Eva Buschmann, Saber Miresmaili, Peter and Tanya Kalyniak, Isidro Ojeda, Nyssa Temmel, Sarah Martz, Yasmin Akhtar, Trish Osterberg and Eduardo Jorel for their help and support.

DEDICATION

For my mother, the symbol of love, and

my father, who wanted to see this day but couldn't...

CO-AUTHORSHIP STATEMENT

Modified versions of Chapters 2 and 4 are manuscripts accepted for publication (Chapter 2 is published; Chapter 4 is in press). I designed the project in consultation with Andrew Riseman and Zamir Punja, and I conducted all laboratory work, data analyses and wrote the manuscripts. A. Riseman provided insights and contributed to the writing.

Chapter 3 is a draft manuscript submitted for publication. I designed the project in consultation with Andrew Riseman, and I conducted all laboratory work, data analyses and wrote the manuscript. A. Riseman provided insights and contributed to the writing.

Chapter 5 is a draft manuscript that will be submitted for publication. I designed the project in consultation with Andrew Riseman and Quentin Cronk, and I conducted all laboratory work, data analyses and wrote the manuscript. A. Riseman provided insights and contributed to the writing.

CHAPTER ONE

INTRODUCTION

1.1 Daphne in the Plant Kingdom

1.1.1 The Family Thymelaeaceae

Daphne L. is a genus within the family Thymelaeaceae Juss. (syns: Aquilariaceae, Daphnaceae, Gonystylaceae, Phaleriaceae, Tepuianthaceae). The family is comprised of 44 genera, approximately 500 species (Watson and Dallwitz, 2007), and has a cosmopolitan distribution with concentrations in tropical Africa, Southeast Asia and Australasia (Watson and Dallwitz, 2007). The plant forms found in this family are mostly trees and shrubs, with a few vines and herbaceous types known. The family is within subclass Dicotyledonae and order Malvales. A few genera within Thymelaeaceae have economic importance as cultivated ornamental shrubs (e.g., *Daphne*, *Dais* L., *Dirca* L. (leatherwood), *Pimelea* Banks ex Gaertn. (rice flower) *Wikstroemia* L.), and for paper production (e.g., *Thymelaea* Mill.) (Watson, 2007; Flora of China, 2005; Soltis and Soltis, 2000). It has also been reported that many species in the family have bioactive compounds e.g. *Daphne genkwa* (Zhou, 1991) and have been used as medicinal plants in Chinese and other Asian cultures. Studies on the chemical profiles of these plants have identified several groups of active compounds including flavonoids, lignans and diterpenes (Zhou, 1991).

The phylogenetic relationships among the genera in Thymelaeaceae have been under extensive discussion recently with various authors proposing a relationship between this family and Euphorbiales, Malvales, and/or Gutiferales (Crawford, 1995; Van der Bank and Fay, 2002). More recently, Thymelaeaceae was investigated by parsimony analysis of 41 *rbcL* (a chloroplast gene) nucleotide sequences, including 27 genera and several outgroup taxa. These results supported the Malvalean placement (Alverson and Baum, 1998). In a second study, Soltis and Soltis (2000) used plastid *trnL* intron sequence analysis that produced highly congruent results to the first *rbcL* results. Therefore, Malvalean placement of this family has been confirmed by both *rbcL* (Alverson and Baum, 1998) and *trnL* (Alverson and Baum, 1998; Bayer and Fay, 1999; Soltis and Soltis, 2000) sequence data. However, within Malvales, the position of Thymelaeaceae remains unresolved because of some characteristic traits of these plants including: plant forms that are mostly shrubs, lianas or herbs (rarely), and non-succulent; leaves that are small to medium-sized, alternate, opposite, or whorled; internal phloem that is present in nearly all genera with secondary thickening developing mostly from a conventional cambial ring. Using both classical and modern data, the accepted systematic placement of Thymelaeaceae is as follows (Watson and Dallwitz, 2007; Flora of China, 2005):

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Malvales

Family: Thymelaeaceae

1.1.2 The Genus *Daphne* L.

The genus *Daphne* is comprised of 95 species distributed primarily in Europe and Asia, with a few species also endemic to Africa and Australia (Flora of China, 2005). Botanically, the petals, which in many familiar flowers form an inner whorl, have all but disappeared in the genus. However, in a few species, the petals are present and represented by a small disk inside the base of the calyx-tube. Within this tube are eight stamens arranged in two whorls of four at different levels. The ovary, which sits at the base, has a short (often absent) style and a fairly obvious (capitate) stigma (Halda, 2001; Mathew et al, 2000).

Based in part on its wide distribution, *Daphne* contains significant variation in morphology, native habitats, and use. Important uses include pharmaceutical, ethnobotanical, and ornamental applications (Zhou, 1991; Brickell and White, 2000a; Mathew, 2000). In horticulture, several species have been commercially introduced because of their many desirable characteristics including attractive foliage, plant habit, flower color and most of all, pleasant fragrance. Propagation of *Daphne* in the horticulture trade can be from seed or cuttings. However, seeds are genetically variable adding undesirable variation to a production (or evaluation) system. Vegetative propagation is more desirable, but traditional cuttings are typically very difficult to root (Brickell and White, 2000b; Matthew, 2000; Chen etal, 1995; Dixon, 1994). For example, *D. cneorum* (Rose daphne, or garland flower), a very fragrant species with good commercial potential, is one of the most popular species in this genus. It forms a low

shrub that, during April and May, bears clusters of fragrant rose-pink flowers (Brickell and White, 2000b). However, it seldom produces seed in cultivation and has to be increased by division, grafting, or cuttings. Unfortunately, rooting percentages experienced by commercial propagators are often between 5% - 20% (Riseman, personal communication). However, as with other crops, the development of *in vitro* propagation protocols can allow for the conservation and production of uniform, disease-free plants (Cohen, 1977; Li and White, 1998; Marks and Simpson, 2000; Sediva, 2002).

1.2 Plant Breeding and Crop Improvement

Prior to the 20th century, plant breeding was largely an art with little or no knowledge of genetic principles (Debener, 1999). However, since the rediscovery of Mendel's seminal work and the application of genetic principles, plant breeding has made enormous gains over the last century. Plant breeders have made significant progress in developing high yielding cultivars, cultivars that can be grown in less favorable soils/environments, and cultivars that are resistant to a variety of pests and diseases. The development of cultivars with disease resistance is one of the major goals for plant breeders due to its direct (e.g., greater yields) and indirect (e.g., reduced pesticide use) importance (Stuber etal, 1999; Fehr, 1987). In general, disease resistance can be thought of as either narrow (i.e., vertical, qualitative), where one or a few major genes condition resistance, or broad (i.e., horizontal, quantitative), where many genes, each with a small contribution, affect resistance (Kozumplinki etal, 2004; Agrios, 2005). Each form has its own advantages and disadvantages. For example, identification and use of vertical resistance is thought of as being easier and more straightforward to manipulate than horizontal resistance.

However, vertical resistance is often specific to only one or a few races of a particular disease while horizontal resistance can be more broadly effective. One basic but critical disadvantage of vertical resistance compared to horizontal resistance is its often short effective lifespan due to pathogen evolution.

1.2.1 Overview of plant improvement strategies

In general, plant improvement is defined as the process of identifying and selecting plants with desirable traits and using them for the production of more plants with favorable traits (Simmonds and Smart, 1999). The production of these plants can be by sexual or asexual systems. Depending on which system is used, various plant improvement strategies are available.

1.2.1.1 *Sexual Reproduction and Plant Breeding*

Sexual reproduction is the primary system by which crops are improved allowing for the recombination of favorable characters into superior individuals. It involves knowledge of the reproductive biology of the crop of interest as well as knowledge of the techniques and procedures required to efficiently identify and recombine the traits of interest. Over the next section, I will review the basic biology of sexual reproduction and how plant breeders work within this context to develop superior germplasm.

Sexual reproduction occurs in the flowers with flower form having significant implications for the plant breeder. Flowers have four major parts: calyx (sepals), corolla (petals), androecium (stamens) and gynaecium (carpel) (Neil, 2006). A flower having all

of these organs is called complete while a flower lacking one or more parts is incomplete. The stamen and carpel are the essential parts of a flower as these organs produce the male and female gametes. A flower that has both of these organs is called perfect while a flower that lacks one of these organs is refered to as imperfect. By definition, perfect flowers are bisexual while imperfect flowers are unisexual (i.e., either pistillate and staminate). Species that bear imperfect flowers on the same plant are called monoecious. However if the staminate and pistillate flowers occur on different individuals, the species is known as dioecious. The mode of reproduction and the ease with which it can be modified by plant breeders largely depends upon these differences, especially with respect to the presence and location of the stamens and pistils (Neal et al, 2006; Neil, 2006).

The morphological features of flowers influence both natural and human directed pollination (i.e., the process of transferring pollen grains from the anther to the stigma). When flower form and development favor pollination within a single flower, the crop is referred to as self-pollinated or an inbreeder. On the other hand, when flower form and development inhibit pollination within a flower and instead, promote inter-flower pollination, the crop is referred to as cross- pollinated or an outcrosser. The impacts of mode of reproduction to the plant breeder are many and are very influential factors in plant breeding and crop improvement practices (Niel, 2006; Neal et al, 2006). However, two of the most important impacts include its effect on the genetic structure of the breeding population and the associated ability to tolerate inbreeding. First, populations of a naturally self-pollinated crop will increase homozygosity across generations while a

cross-pollinated crop will naturally increase the level of heterozygosity across generations. Second, a self-pollinated crop is normally tolerant of inbreeding while a cross-pollinated crop is typically intolerant of inbreeding often suffering from inbreeding depression after a few generations of self-pollination. In addition to these impacts, the mode of reproduction can be exploited by the plant breeder to help them better manage and manipulate the genetics of the population. For example, a self-pollinated crop breeder can easily increase homozygosity and inbred line development by simply allowing pollination to occur naturally. Following an initial cross-pollination generation (i.e., labor intensive step), 6-8 generations of natural self-pollination will produce a functional level of homozygosity and sufficient uniformity for commercialization.

1.2.1.2 Asexual/Clonal Reproduction

Although sexual reproduction is the primary way plant breeders manipulate genetic variation and create new cultivars, it is not the only way. Asexual or clonal reproduction is a valuable tool that both allows for mass production of individual genotypes but also as an important source of genetic variation. In many crops where sexual reproduction and/or the level of variation is problematic for cultivar development (e.g., low fecundity, inbreeding depression, low genetic diversity), clonal reproduction can be of value. Specifically, clonal reproduction can allow plant breeders to exploit naturally occurring somatic mutations (e.g., sports), somatic mutations induced by chemical or radiation treatments, or somaclonal variation induced via tissue culture technologies. Even for crops that form adequate quantities of seed for commercial production (e.g., diploid seed

geraniums), asexual propagation may be of value for specific applications (e.g., bacterial/viral indexing) or germplasm types (e.g., tetraploid geraniums)

1.2.2 Breeding of Ornamental Plants

1.2.2.1 Overview

Many cultures around the world use flowers and ornamental plants to decorate and celebrate major events, symbols of beauty and respect, and objects of art. When this practice began, most ornamental plants were wild collected and used directly as they occurred in nature. As time progressed to the Victorian era, gardening and ornamental plants reached its pinnacle in terms of social status. Great effort was extended to collect the most unusual and unique specimens. At the same time, gardeners started to generate non-naturally occurring forms for use in this budding industry. Works of art from this period provide us with a rich accounting of the numerous flowers and cultivars common at that time, most of which were either natural mutants clonally propagated or the result of 'happenstance' breeding. The vast majority of plants grown at this time were ones that could be easily propagated either by seed (e.g., annuals) or by asexual propagation (e.g., cutting, divisions, bulblets, etc). To help support this growing interest in gardening, commercial horticulture firms formed and became a very powerful industry. For example the Dutch bulb industry, which started its work by introducing tulip bulbs imported from today's Turkey, is currently providing 93% of the world's bulbs with over nine billion bulbs sold per year (Perry, personal communications,). Over time, many seed and vegetativly propagated cultivars have been preserved as important sources of germplasm and are now known as heirloom or heritage cultivars. In the modern

ornamental plant breeding industry, tools involving molecular biology are commonly used for the identification and selection of improved cultivars (Harrera, 1992). In addition, modern ornamental plant breeders often rely on modern greenhouses and nurseries to help maintain environmental conditions at optimum levels thereby allowing for maximum growth potential to be observed among breeding selections.

1.2.2.2 Daphne Breeding and Improvement

During the last 25-30 years, there has been a surge of activity among horticulturists to produce new and superior hybrids through classical breeding methods. However, sexual hybridization within and among species of *Daphne* has been either unsuccessful (i.e., no fruit), or the hybrids produced were of low fertility or sterile (Brickell and White, 2000b). Because of this difficulty in reliably producing seeds/hybrid progeny and the need for increased uniformity during production, *Daphne* plants are primarily propagated for commercial (or research) use via asexual propagation. Unfortunately, *Daphne* cuttings are typically very difficult to root (Brickell and White, 2000a; Matthew etal, 2000; Chen etal, 1995; Dixon, 1994). Typical rooting percentages of the most common daphne in the horticulture trade, *D. cneorum*, are below 30% and are limited to a three month window (i.e., May-July) during the year. In addition, *Daphne* stock plants have been observed to decline over time yielding fewer and fewer cuttings. Therefore, the development of micropropagation protocols is seen as a way to address these limitations by allowing for the year-round production of genetically uniform, disease-free plants for use (Cohen, 1977; Li and White, 1998; Marks and Simpson, 2000; Sediva, 2002).

1.2.3 Breeding for Important Traits in Horticultural Crops

1.2.3.1 Overview

When horticulture firms first started to breed ornamental crops, the most important characters identified included fragrance, plant form and morphology, and leaf and flower colour, all associated with the aesthetics of the plant. For many years, these remained the primary traits of interest. For example, initial rose breeding efforts were directed at increasing the range of colors, plant forms, and flower size (Debener, 1999). However, more recently, breeding for resistance to environmental stressors, both biotic and abiotic has become a priority for many plant breeding programs (Agrios, 2005; Ashraf and Harris, 2005). Specifically, traits of modern interest include fungal/bacterial disease resistance, insect resistance, and drought and cold tolerance; all non-aesthetic traits but important for sustainable production. Breeding disease resistant cultivars has received more attention in recent years and has been greatly helped by advances in genetics and other related sciences. With the heightened awareness of the environment and sustainability, the incorporation of these traits is vital to the future of this industry and the success of many crops (Agrios, 2005; Neil, 2006).

1.2.3.2 Breeding for resistance to abiotic and biotic stressors

Plant stress experienced by plants can be divided into two groups: abiotic and biotic. Abiotic stressors are derived from interactions between the plant and its physical environment while biotic stressors involve the interaction between two organisms, the plant and the pest, (Yeo and Flowers, 1989; Ashraf and Harris, 2005). Abiotic stressors include the harmful effects of salinity, drought, metal toxicity, nutrient deficiency and/or

temperature extremes. For example, salinity stress occurs when there is an excessive amount of soluble salts in the soil that negatively affect the physiology of plant. It can be quantified as electrical conductivity (EC), exchangeable sodium percentage (ESP), or sodium adsorption ratio (SAR) (Yeo, 1989; Ashraf and Harris, 2005). In addition, abiotic stressors can include air pollution, wind, shade, or any shortage in an essential resource that affects plant growth e.g., gaseous or light sources. In some cases, such as the supply of water, either too little (drought) or too much (flooding) can cause stress on the plant (Ashraf and Harris, 2005). In addition, abiotic and biotic stressors can interact magnifying their individual effects. For example, plants suffering from nutrient deficiency are generally more susceptible to pathogens (Ashraf and Harris, 2005).

1.2.3.2.1 Breeding for resistance to abiotic stressors

Plant resistance to abiotic stressors in nature evolved through stress avoidance or stress tolerance mechanisms. Avoidance enables the plant to avoid exposure to the stressor by exclusion or minimizing the damage caused. Avoidance mechanisms are diverse and depend on the type of stress. They can take place in the whole plant, an individual organ, or at the cellular level. Stress tolerance refers to the ability of the plant to maintain normal function in the presence of the stressor or the damage caused by a stressor (Ashraf and Harris, 2005). In the recent years, many cultivars of plants resistant to individual stressors have been developed through traditional plant breeding, asexual selection, or other more technological methods e.g., recombinant DNA (Yeo, 1989; Ashraf and Harris, 2005).

1.2.3.2.2 Breeding for resistance to biotic stressors

Plants are constantly being challenged by pathogens and a wide range of control strategies (e.g., chemical control, environmental manipulation) have been developed to control them. Although these methods are somewhat efficient in controlling the negative impacts of these pathogens in the short term, they do not lead to long-term sustainability. For example, reliance on fungicides/pesticides is expensive, environmentally damaging, and potentially hazardous for human health. On the other hand, developing cultivars with host-plant resistance to these pests can provide a long-lasting and sustainable solution. While the incorporation of genetic resistance into plants is a lengthy and expensive process, it is deemed relatively inexpensive compared to the costs associated with not pursuing this goal (Neal et al, 2006).

Many authors have suggested that the differences between horizental and vertical resistance are artificial and question if horizontal resistance truly exists (Agrios, 2005). Future research will examine this issue and will hopefully elucidate the situation more clearly. In the meantime, discussions on this topic as related to the mechanisms of disease resistance and the genetic control of resistance continue (Agrios, 2005; Neal et al, 2006; Johnson, 1978).

Development of pathogen resistant cultivars requires a thorough understanding of the evolutionary interrelationships between plants and their pathogens. The co-evolution of host and pathogen can not be understood without knowledge relating to the pathogen including its pathogenecity (i.e. the ability the pathogen to display virulence and induce

disease on a given host). The efficiency of developing resistant cultivars depends on this understanding and the exploitation of the interactions between host and pathogen (Neal etal, 2006; Agrios, 2005). In contrast to breeding for yield or other horticultural traits, the development of cultivars with pathogen resistance is more complex because it involves the interaction between two dynamic organisms (Neal etal, 2006; Agrios, 2005). The process of developing these resistant types typically involves a type of germplasm screen that allows for the identification of genotypes possessing resistance against a specific pathogen.

1.3 Plant Fungal Diseases

The majority of the 100,000 described fungi, are saprophytic and not pathogens (Agrios, 2005). However, approximately 10% are considered plant pathogens and may cause disease on many plant species (Agrios, 2005). Fungal pathogens, based on their life style, are categorized into two major groups: obligate and non-obligate. Obligate fungi only grow and complete their life-cycle on living hosts while non-obligate pathogens need a living host for only part of their life cycle, completing the remainder of their life cycle on dead material. The capacity of pathogen to incite disease among members of a host species is called 'pathogenicity' and when it is able to induce symptoms of disease, the pathogen is termed 'virulent'. The term 'avirulent', often used to describe lack of pathogenicity of a certain isolate, suggests an internal failure of a pathogen to be able to induce disease on the host (Agrios, 2005; Johnson, 1978).

1.3.1 Disease Development

1.3.1.1 Process of infection and disease progression

Three basic factors are involved in the development of disease; the pathogen, the host plant, and the environment surrounding these two interacting organisms. If any one of these factors is lacking or not promotive of disease (e.g., proper conditions for disease development), disease cannot develop. Every disease needs a specific range of environmental conditions (e.g., favorable temperature, proper humidity) to develop (Agrios, 2005). In addition to this three-way interaction, the genetic interaction between host plant and pathogen plays a crucial role in disease development. The concept of 'parallel evolution' for the compatibility between the host and pathogen genomes, first introduced in the mid 1950's (Flor, 1955), is generally accepted by most plant pathologists today (Agrios, 2005). In support of this concept, it has been shown that there is a balance between pathogen virulence and host plant resistance (Agrios, 2005). The concept of 'gene-for-gene' (Flor, 1955) describes the interaction between a pathogen and host where a balance exists between a pathogen's gene required for virulence and a host's gene responsible for resistance. Based on this theory, for every virulent gene in a pathogen's genome, there is a corresponding resistance gene in the host (Flor, 1955; Agrios, 2005). Generally, these genes are active during different stages of disease development (Agrios, 2005; Johnson, 1978; Flor, 1955).

The process of disease development involves many individual events based on the interaction between pathogen and host plant. The major events in this process are:

inoculation, penetration, infection, invasion (colonization), growth, multiplication, and spread of the pathogen (Agrios, 2005). Inoculation, the initial contact between plant and pathogen, may involve two steps i.e. attachment and germination (Osherov and May, 2001; Agrios, 2005). However, for some pathogens, additional steps are present and include specific attachment processes (adhesion/ bond) and/or thigmotropism (surface sensing). Initial adhesion of the spore to the plant cuticle is commonly based on hydrophobic interactions. Once securely attached, the spore germinates and produces a germ tube for penetration of the plant's cuticle. Usually, spore germination is affected by the amount of nutrients (e.g., sugars, potassium, calcium and amino acids) exuded or cutin monomers from the plant (Osherov and May, 2001; Agrios, 2005). Pathogens most often penetrate the plant surface through natural openings such as stomata or through wounds. In addition, some pathogens (e.g., rust fungi, *Uromyces* sp.) can sense appropriate points of entry on the plant (Osherov and May, 2001; Agrios, 2005). Other pathogens do not use existing openings but instead, penetrate plant surfaces through direct action. These pathogens can penetrate through a plant surface by using either physical (e.g., appressorium) or enzymatic (i.e., cell wall degrading enzymes including cellulase, pectinase and cutinase) forces. However, successful penetration does not always lead to infection. Successful infection relies on the pathogen's contact with susceptible cells and tissues of the host. During infection, fungal pathogens enter plant cells in order to derive nutrition. As nutrients are made available, the pathogen grows and multiplies within these tissues leading to more widespread invasion of the plant (Osherov and May, 2001; Agrios, 2005).

One of the fundamental problems associated with controlling fungal pathogens is their ability to easily infect neighboring plants. Fungi use a range of vectors to gain access to new hosts including water (e.g., overhead irrigation), air flow (e.g., horizontal air flow fans), insects, and/or humans (e.g., contaminated secateurs) (Agrios, 2005). In addition, infection can occur on different plant parts/organs with each pathogen specialized for a specific host. For example, some root pathogens (e.g., *Fusarium* spp., *Phytophtora* spp. *Thielaviopsis basicola*) penetrate the plant surface through lateral roots and degrade root tissues using specific enzymes (Agrios, 2005; Rothrock, 1992; Hood and Shew, 1997a).

1.3.1.2 Plant Defense Systems

Most plants are resistant to most of the pathogens found in their environment although each plant may be attacked by many pathogens every day (Osherov and May, 2001; Agrios, 2005). While these plants will suffer to some extent from these pathogens, many survive and live for long periods (Agrios, 2005). The failure of most pathogens to infect plants other than their host indicates that plants can generally defend themselves against pathogens (Agrios, 2005). In these plants, defense against pathogen attack can be based on either physical or biochemical barriers. The physical or structural defense system works as a barrier against pathogen intrusion while the biochemical system can generate compounds toxic to the pathogen or able to prevent its growth. These systems can be either pre-existing or induced, with different plants using various combinations based on their interactions with their pathogen (Agrios, 2005). The first line of defense for many plants is their pre-existing structural defense system that may include waxes, a thick cuticle layer, and/or cell walls. Plants may have a pre-existing biochemical defense

system, such as the use of phenolic compounds, which can inhibit pathogen growth and multiplication (Agrios, 2005). While these primary defense systems are essential, in many cases of disease, pathogens can easily overcome these pre-existing barriers to reach the internal tissues. To augment the pre-existing defense systems, many plants are able to induce a secondary defense system after detection of a pathogen. These systems are typically more targeted and intense defense strategies against the pathogen. Some induced defense systems are structural and include cytoplasmic defense reaction, cell wall defense reactions, and hypersensitive defense reactions (Agrios, 2005). In cytoplasmic defense reactions, the cytoplasm is modified into a granular and dense matrix thereby inhibiting fungal growth. Cell wall defense reactions may include a thickening of the cell wall in the areas around sites of penetration (Agrios, 2005). This reaction can lead to the death of invaded cells i.e. a necrotic or hypersensitive defense reaction. In addition, specific biochemicals (e.g., phytoalexins; very toxic antimicrobial compounds) can be produced following infection as part of an induced defense system. In all induced defense mechanisms, early recognition of the pathogen by the host plays a vital role in successful defense.

1.3.2 *Thielaviopsis basicola*; a Fungal Root Pathogen

1.3.2.1 Overview

Some plant pathogens are specialized for life in the soil and have special abilities to infect plants via their root systems (Hood and Shew, 1997a; Agrios, 2005). Typically, symptoms induced by these pathogens first appear on the roots as water soaked regions

that later discolor and become necrotic. Root pathogens are mostly non-obligate parasites that can live and grow in the soil as without a host using organic material (e.g., dead plant tissue) available in the soil. These fungi typically prefer soils with high moisture content and a high atmospheric relative humidity. Many of the most aggressive, and economically important, plant pathogens are among this group and include *Fusarium* spp., *Rhizoctonia* spp., *Phytophthora* spp., and *Thielaviopsis basicola* (Agrios, 2005; Hood and Shew, 1997b).

1.3.2.2 Thielaviopsis basicola: Taxonomy and Pathogenicity

Thielaviopsis basicola (Berk & Br.) Ferr. (synanamorph *Chalara elegans,* Nag Raj and Kendrick; *Torula basicola* Berk. & Broome; *Trichocladium basicola* (Berk. & Broome) J.W. Carmich.) is a widespread root fungal pathogen found in both agricultural and nonagricultural soils (Trojak-Goluch and Berbec, 2005; Hood and Shew, 1996). A wide range of economically important plants are infected by *T. basicola* including carrot (*Daucus carota* L.) (Punja et al, 1992), cotton (*Gossypium hirsutum* L.) (Wheeler et al, 1999), and tobacco (*Nicotiana tabacum* L.) (Hood and Shew, 1996 & 1997b). Infection by *T. basicola* commonly results in a form of black root rot disease, whole plant stunting, and delayed maturity, all of which reduce yield and crop quality. This pathogen is generally considered a facultative parasite, recently described (Walker etal, 1999; Hood and Shew, 1997b) as a hemibiotrophic pathogen. This designation refers to *T. basicola*'s ability to survive for long periods of time outside of a living host by saprophytic consumption of soil organic matter (Hood and Shew, 1997b; Punja et al, 1993). The fungus produces two spore types, phialospores (endoconidia) and

chlamydospores (aleuriospores). The chlamydospore is characterized by a thick pigmented wall and divisions into compartments or segments, each of which is able to germinate. The phialospores are hyaline, not septate, and have a rectangular shape. The taxonomic classification of *T. basicola* is as follows (Nag Raj and Kendrick, 1975):

Kingdom: Fungi

Division: Eumycota

Subdivision: Deuteromycotina (Fungi Imperfecti)

Class: Moniliales

Genus: *Chalara*

Species: *Thielaviopsis basicola* (syn. *Chalara elegans*)

The literature indicates that variability in virulence, due to both external and internal factors, exists within *T. basicola.* For example, *T. basicola* is reported to be most aggressive during the growing season when soil temperatures are ≤ 24°C (Walker et al, 1999) and soil water content is high (Rothrock, 1992; Walker et al, 1999). Bottacin et al. (1994) described variation in virulence among several isolates of *T. basicola* and related this to their genetic composition. Specifically, they described the existence of dsRNAs in some, but not all, isolates of *T. basicola* (Bottacin et al., 1994) and related this to changes in the level of virulence (Park et al, 2005; Punja et al, 1992; Geldenhius et al, 2004). In support, it has been shown that strains without dsRNA were less pathogenic than strains with one dsRNA strand and that these were more pathogenic than strains with more than one dsRNA strand (Punja, 1995). As a result, the authors have concluded that the

presence of this particular dsRNA in *T. basicola* increases the pathogenicity of this fungus (Punja et al, 1992; Park et al, 2005). Most recently, Park et al (2005) examined infection of *T. basicola* by two totivirus-like dsRNAs. A full-length cDNA clone was developed from the 5.3 kb dsRNA element present. Sequence analysis revealed that it contained three large putative open reading frames (ORFs). These results indicate that the presence of putative virus-like particles in the cytoplasm, which were similar in both shape and size to viruses in the Totiviridae, increase the virulence in certain isolates of *T. basicola* (Punja, 1995; Park et al, 2005).

Observations on the interactions of *T. basicola* with other organisms show that *T. basicola* can be sensitive to antagonism (Howell, 2003, Hood and Shew, 1997b). For example, *Pseudomonas* spp. populations in the rhizosphere of tobacco roots produced biocontrol compounds (e.g., hydrogen cyanide [HCN] suppressive to *T. basicola* (Troxlert and Berling, 1997). The antagonistic activity of *Trichoderma* spp. against many pathogens, including *T. basicola*, have also been reported. However, biocontrol using *Trichoderma* against *T. basicola* has not yet been approved for commercial applications (Howell, 2003). In another study, a synergism between the root-knot nematode, *Meloidogyne incognita* (Kofoid & White), and *T. basicola,* was identified. When occurring together, these pests greatly facilitate the development of black root rot in cotton; a seedling disease that is characterized by severe rotting of the cortex of young cotton hypocotyls and roots (Walker etal, 1999).

1.4 Daphne Diseases

1.4.1 Overview

Daphne species are well-known because of their delightful scent and attractive flowers. However, many problems have been associated with growing these plants by both commercial growers and Daphne enthusiasts. Reports indicate that one of the major limitations to Daphne's survival in cultivation is susceptibility to fungal root pathogens that cause root rot and chlorosis (Linderman and Toussoun, 1967). Previously, pathology reports have identified several pathogens as possible causal agents including *Fusarium* spp. (Pataky, 1988), *Phytophthora cactorum* (Lebert& Cohn) J. Schroeter (Linderman and Zeitoun, 1977; Anonymous-USDA, 1960), *P. nicotiana* var. *parasitica* (Breda de Haan) Tucker (Tompkins, 1951), and *Pythium* spp. (Grand, 1985), as well as several unidentified fungal pathogens. In addition to fungal pathogens, many viral diseases have been reported in *Daphne*. These viruses have been categorized into three groups based on mode of transmission: aphid-borne viruses, nematode-borne viruses, and contact spread viruses (Moran, 1995; Halda, 2001). Although many of these viruses remain unknown, several that have been confirmed include Alfalfa Mosaic Virus, Cucumber Mosaic Virus, Daphne Virus S and Y, Arabis Mosaic Virus, Tobacco Ringspot Virus, Daphne Latent Ringspot Virus, and Carnation Mottle Virus (Moran, 1995; Halda, 2001).

1.4.2 Daphne Sudden Death Syndrome

A disease, refered to as 'Daphne Sudden Death Syndrome' (DSDS) or 'Mad Daphne Disease' by growers and gardening enthusiasts, kills the plant, as the name suggests, very quickly. The symptoms are to some extent different from those previously reported on

Daphne. In this case, diseased plants developed black lesions on their roots and died within 2-3 weeks after the first appearance of foliar symptoms (i.e., leaf chlorosis). Once foliar symptoms are seen, plant death is imminent. The key symptoms of this disease are the brown to black necrotic lesions on the roots, leaf chlorosis leading to abscission, stunting, and whole plant collapse.

1.5 Objectives

Although there is a great amount of passion directed towards *Daphne* species and their cultivation, very few peer-reviewed publications exist that address their pathology, phylogeny, or crop improvement techniques (e.g., breeding and biotechnology). This research addresses several fundamental questions as they relate to *Daphne* disease and the development of improved cultivars. The main objectives of this research were:

1) To identify the causal agent(s) of Daphne Sudden Death Syndrome (DSDS) affecting *Daphne* cultivars grown in British Columbia;

2) To develop efficient *in vitro* micropropagation protocols for selected *Daphne* species;

3) To develop a robust germplasm screen to identify variation in taxon x pathogen interactions;

4) To develop a phylogeny for selected *Daphne* species based on DNA sequence information and associate DSDS resistance to clade structure.

1.6 References

Agrios GN (2005) Plant Pathology. 5th ed. Academic Press, Elsevier Science and Technology books, NY.

Alverson WS and Baum D (1998) Circumscription of the Malvales and relationships to other Rosidae. Amer. J. Bot. 85, 876-877.

Anonymous (1960) Index of plant diseases in the United States, U.S. Department of Agriculture. Handbook No.165. p. 531. Washington DC.

Ashraf M and Harris P (2005) Abiotic stresses : plant resistance through breeding and molecular approaches. The Haworth Press, Inc., Binghamton, NY.

Bayer C and Fay M (1999) Support for an expanded concept of Malvaceae within recircumscribed order Malvales: a combined analysis of plastid *atpB* and *rbcL* DNA sequences. Bot. J. Linn. Soc. 129, 267-303.

Bottacin AM, Levesque CA and Punja ZK (1994) Characterization of dsRNA in *Chalara elegans* and effects on growth and virulence. Phytopathology. 84, 303 -312.

Brickell CD and White R (2000a) A quartet of New Daphnes. The New Plantsman. March, 6-18. The Royal Horticultural Society, London.

Brickell CD and White R (2000b) Further trio of new Daphne hybrids. The New Plantsman. December, 236-248. The Royal Horticultural Society, London.

Chen DZ, Liu T and Li Y (1995) Studies on rapid clonal propagation of *Daphne odora* in vitro culture. Acta Hort. 404, 15-20.

Cohen D (1977) Thermotherapy and meristem-tip culture of some ornamental plants. Acta Hort. 78, 381-387.

Crawford DJ (1995). Plant Molecular Systematics, Macromolecular Approaches, Wiley-Interscience publication, New York, NY.

Dixon RA (1994) Plant Cell Culture, A Practical Approach. Oxford University Press. Oxford [England]; New York: IRL Press at Oxford University Press, c1994.

Debener T (1999) Genetic analysis of horticulturally important morphological and physiological characters in diploid roses. Gartenbauwissenschaft. 64 (1) 14–20.

Fehr WR (1987) Principles of cultivar development. Macmillan Publishing Co. New York.

Flor H (1955) Host-parasite interactions in flax rust-its genetics and other implications. Phytopathology. 45, 680–685.

Flora of China (2005) Vol. 13 [*Clusiaceae through Araliaceae*]. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Geldenhius M, Roux J, Wingfield M and Wingfield B (2004) Development of polymorphic markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR. Mol. Eco. Notes. 4, 547–550.

Grand LF (1985) North Carolina Plant Disease Index. North Carolina Agri. Res. Ser. Tech. Bul. 240, 1-157.

Halda JJ (2001) The Genus *Daphne*. Eva Kucerova- Sen Dobre Publikaca, Prague, Czeck Republic.

Harrera J (1992) Systematics and evolution flower variation and breeding systems in the Cistaceae. Plant Syst. Evol. 179, 245-255.

Hood ME and Shew HD (1996) Pathogenesis of *Thielaviopsis basicola* on a susceptible and a resistant cultivar of burley tobacco. Phytopathology. 86, 38-44.

Hood ME and Shew HD (1997a) Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs. Phytopathology. 87, 228-235.

Hood ME and Shew HD (1997b) Reassement of the role of saprophytic activity in the ecology of *Thielaviopsis basicola.* Phytopathology. 87, 1214- 1219.

Howell C (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Disease. 87 (1), 4-10.

Johnson R (1978) Practical breeding for durable resistance diseases in self-pollinating cereals. Euphyt. 27, 529-540.

Kozumplinki V, Barlei M and Barkie I (2004) Plant breeding and environment. Agri. Consp. Sci. 69, 67-75.

Li H and White D (1998) Biological control of *Botrytis*, *Phytophtora* and *Pythium* by *Bacillus subtilis* of micropropagated plants. Plant Cell, Tis. Org. Cult. 52, 109-112.

Linderman RG and Zeitoun F (1977) *Phytophthora* causing root rot and wilt of nursery-grown native Western azalea and other ornamental plants. Plant Dis. Rep. Vol.16, No.12.

Linderman RG and Toussoun TA (1967) Behavior of chlamydospores and endoconidia of *Thielaviopsis basicola* in nonsterilized soil. Phytopathology. 57, 729-731.

Marks T and Simpson S (2000) Interaction of explant type and indol-3-butyric acid during rooting in vitro in a range of difficult and easy to root woody plants. Plant Cell Tiss. Org. Cult. 62, 65-74.

Mathew B, Brickell C and White R (2000). The smaller Daphnes. The Proceeding of the Conference " Daphne 2000 " April 2000. The Royal Horticultural Society, London.

Moran J (1995) Daphne viral diseases. Agri. Notes 175, 1-3, State of Victoria, Department of Primary Industeries, Melbourne, Australia.

Nag Raj T and Kendrick B (1975) A Monograph of *Chalara* and Allied Genera. Wilfred Laurier Universiiy Press. Waterloo, Ontario, Canada.

Neal C, Stoskopf D, Tomes T and Christie B (2006) Plant Breeding: Theory and practice. Westview Press. Reprint. Jodhpur, Scientific.

Neil A (2006) Introduction to Flower Breeding & Genetics; Issues, challenges, and opportunities for the 21st Century. Springer, St. Paul, MN, USA.

Osherov N and May GS (2001) The molecular mechanisms of conidial germination. Microbiol. Lett. 199 153–160.

Park Y, James D and Punja ZK (2005) Co-infection by two distinct totivirus-like double-stranded RNA elements in *Chalara elegans* (*Thielaviopsis basicola*). Virus Res. 109, 71–85.

Pataky NR (1988) Report on Plant Disease. RPD No. 650, Department of Crop Sciences, University of Illinois at Urbana-Champaign.

Punja ZK (1993) Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*. Can. J. Bot. 71, 447-456.

Punja ZK (1995) Influence of double-stranded RNAs on growth, sporulation, pathogenicity, and survival of *Chalara elegans*. Can. J. Bot. 73, 1001-1009.

Punja ZK, Chittaranjan, S and Gaye, M.M (1992) Development of black root rot caused by *Chalara elegans* on fresh market carrots. Can. J. Plant Path. 14, 299-309.

Rothrock CS (1992) Influence of soil temperature, water, and texture on *Thielaviopsis basicola* and black root rot of cotton. Phytopathology.82, 1202-1206.

Sediva J (2002) Using micropropagation for Daphne conservation (*Daphne cneorum* L.) in the Czech Republic. Acta Pruhoniciana. 2002, No.73, 52-59.

Simmonds N and Smart J (2000) Principles of crop improvement. Second Edition. University of Southampton press, UK.

Soltis DE and Soltis P S (2000) Angiosperm phylogeny inferred from 18s rDNA, *rbcL*, and *atpB* sequences. Bot. J. Linn. Soc.133, 381-461.

Stuber C, Polacco M and Senior L (1999) Synergy of empirical breeding, marker-assisted selection, and genomics to increase crop yield potential. Crop Sci. 39, 1571–1583.

Tompkins CM (1951) Stem and root rot of *Daphne odora* caused by *Phytophthora parasitica*. Phytopathology. 41, 654-656.

Trojak-Goluch A and Berbec A (2007) Meiosis and fertility in interspecific hybrids of *Nicotiana tabacum* L., *N. glauca* Grah. and their derivatives. Plant Breeding 126, 201-206.

Troxler J and berling C (1997) Interactions between the biocontrol agent *Pseudomonas fluorescens* CHA0 and *Thielaviopsis basicola* in tobacco roots observed by immunofluorescence microscopy. Plant Path. 46, 62-71.

Van der Bank M and Fay M F (2002) Molecular phylogeny of *Thymelaeaceae* with particular reference to African and Australian genera. Taxon. 51, 329-339.

Walker N, Kirkpatrick T, and Rothrock C (1999) Effect of temperature on and histopathology of the interaction between *Meloidogyne incognita* and *Thielaviopsis basicola* on cotton. Phytopathology. 89 613-617.

Watson L and Dallwitz MJ (2007) The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Delta press, Albany, Australia.

Wheeler TA, Gannaway JR and Keating K (1999) Identification of resistance to *Thielaviopsis basicola* in diploid cotton. Plant Disease. 83, 831-833.

Yeo AR and Flowers TJ (1989) Selection for physiological characters-examples from breeding for salt tolerance. Plants under stress biochemistry, physiology and ecology and their application to plant improvement. Cambridge University Press.

Zhou B (1991) Some progress on the chemistry of natural bioactive terpenoids from Chinese medicinal plants. Memorias do instituto Oswaldo Cruz. 86 (suppl. 2) 219-226.

CHAPTER TWO [1]

First report of *Thielaviopsis basicola* on *Daphne L.*

2.1 Introduction

Rose daphne or garland flower (*Daphne cneorum* L.) is one of the most popular perennial flower species among discriminating ornamental plant growers. Daphne's appeal is based on many desirable characteristics, including its attractive foliage, variable plant habits and flower colors, but most of all, its sweet fragrance or perfume. However, due to problems reported by commercial growers and homeowners, daphne has acquired a poor reputation for long-term performance. Reports indicate that one of the major limitations to daphne's survival in cultivation is susceptibility to fungal root pathogens.

Previously, reports have identified *Fusarium* sp. (Pataky, 1988), *Phytophthora cactorum* (Lebert& Cohn) J. Schroeter (Linderman and Zeitoun, 1977), *P. nicotiana* var. *parasitica* (Breda de Haan) Tucker (Tompkins, 1951), and *Pythium sp.* (Grand, 1985), as well as several unidentified fungal pathogens, as possible causal agents of this problem in

---

[1] A version of this chapter has been published.

Noshad D, Punja ZK, Riseman A, 2006. First report of *Thielaviopsis basicola* on *Daphne L.* Canadian Journal of Plant Pathology 28, 310-312.

different regions of the USA. In 2001, symptoms of an undescribed daphne disease were reported in Vancouver, British Columbia. Typical symptoms were somewhat inconsistent from those in previous pathology reports on daphne in that these plants all had black lesions on the roots and died within 2 weeks following appearance of the first foliar symptoms. This disease, coined 'Daphne Sudden Death Syndrome' (DSDS) or 'Mad Daphne Disease' by gardening enthusiasts, kills plants, as the names suggests, quickly following the first foliar symptoms. Evaluation of DSDS indicates the following progression of symptoms: (i) brown to black necrotic lesions on the roots, (ii) leaf chlorosis leading to abscission, (iii) whole plant stunting, and (iv) stem collapse and plant death.

The sypotoms described are consistent with infection by *Thielaviopsis basicola* (Berk. & Br.) Ferraris (syn. *Chalara elegans* Nag Raj et Kendrick). In Canada, this pathogen causes the disease 'black root-rot' on crops such as carrot (*Daucus carota* L.) (Punja et al., 1992) and tobacco (*Nicotiana tabacum* L.) (Gayed 1972; Stover 1950a, 1950b) while also found on several ornamental species such as poinsettia (*Euphorbia pulcherrima* (Willd. ex Klotzsch) Graham) and petunia (*Petunia hybrida* Vilm.) (Punja et al., 1992). In addition, it has been reported to parasitize important agricultural crops including cotton (*Gossypium* spp.), beans, (*Phaseolus vulgaris* L.), pansy (*Viola tricolor* L.), and peanuts (*Arachis hypogaea* L.) (Hood and Shew, 1997a).

Thielaviopsis basicola is a widespread root fungal pathogen found in both agricultural and nonagricultural soils (Trojak-Goluch and Berbec, 2005; Hood and Shew, 1996). A

wide range of economically important plants are infected by *T. basicola* including carrot (*Daucus carota* L.) (Punja etal, 1992), cotton (*Gossypium hirsutum* L.) (Wheeler etal, 1999), and tobacco (*Nicotiana tabacum* L.) (Hood and Shew, 1997b & 1996). Infection by *T. basicola* commonly results in a form of black root rot disease, whole plant stunting, and delayed maturity, all of which reduce yield and crop quality. This pathogen is generally considered a facultative parasite recently, has been described (Walker etal, 1999; Hood and Shew, 1997a&b) as a hemibiotrophic pathogen. This designation refers to *T. basicola*'s ability to survive for long periods of time outside of a living host by saprophytic consumption of soil organic matter (Hood and Shew, 1997b; Punja etal, 1993). The fungus produces two spore types, phialospores (endoconidia) and chlamydospores (aleuriospores). The chlamydospore is characterized by a thick pigmented wall and divisions into compartments or segments, each of which is able to germinate. The phialospores are hyaline, not septate, and have a rectangular shape. The taxonomic classification of *T. basicola* is as follows (Nag Raj and Kendrick, 1975):

Kingdom: Fungi

Division: Eumycota

Subdivision: Deuteromycotina (Fungi Imperfecti)

Class: Moniliales

Genus: *Chalara*

Species: *Thielaviopsis basicola* (syn. *Chalara elegans*)

The literature indicates that variability in virulence, due to both external and internal factors, exists within *T. basicola*. For example, *T. basicola* is reported to be most aggressive during the growing season when soil temperatures are ≤ 24°C (Walker etal, 1999) and soil water content is high (Rothrock, 1992; Walker et al, 1999). Bottacin et al. (1994) described variation in virulence among several isolates of *T. basicola* and related this to their genetic composition. Specifically, they described the existence of dsRNAs in some, but not all, isolates of *T. basicola* (Bottacin et al., 1994) and related this to changes in the level of virulence (Park etal, 2005; Punja etal, 1995; Geldenhius etal, 2004). In support, it has been shown that strains without dsRNA were less pathogenic than strains with one dsRNA strand and that these were more pathogenic than strains with more than one dsRNA strand (Punja, 1995). As a result, the authors have concluded that the presence of this particular dsRNA in *T. basicola* increases the pathogenicity of this fungus (Punja, 1995; Park etal, 2005). Most recently, Park et al (2005) examined infection of *T. basicola* by two totivirus-like dsRNAs. A full-length cDNA clone was developed from the 5.3 kb dsRNA element present. Sequence analysis revealed that it contained three large putative open reading frames (ORFs). These results indicate that the presence of putative virus-like particles in the cytoplasm, which were similar in both shape and size to viruses in the Totiviridae, increase the virulence in certain isolates of *T. basicola* (Punja, 1995; Park etal, 2005; Wattimena, 2001).

Observations on the interactions of *T. basicola* with other organisms show that *T. basicola* can be sensitive to antagonism (Howell, 2003, Hood and Shew, 1997b). For example, *Pseudomonas* spp. populations in the rhizosphere of tobacco roots produced

biocontrol compounds (e.g., hydrogen cyanide (HCN)) suppressive to *T. basicola* (Troxlert and berling, 1997). The antagonistic activity of *Trichoderma* spp. against many pathogens, including *T. basicola*, have also been reported (Howell etal, 2003). However, biocontrol using *Trichoderma* against *T. basicola* has not yet been approved for commercial applications (Howell etal, 2003). In another study, a synergism between the root-knot nematode, *Meloidogyne incognita* (Kofoid & White), and *T. basicola,* was identified. When occurring together, these pests greatly facilitate the development of black root rot in cotton; a seedling disease that is characterized by severe rotting of the cortex of young cotton hypocotyls and roots (Walker etal, 1999). Evaluating genetic variation in *T. basicola* using ISSR-PCR, seven of fourteen primer pairs resulted in the amplification of single polymorphic fragment indicating quantifiable variation is present among populations (Geldenhuis etal., 2004). Continued use of these primers will enable further molecular characterization of this important pathogen resulting in an enhanced understanding of its population structure (Geldenhuis et al, 2004).

Little is known about the factors (e.g., cultural conditions, host plant genetics) that affect DSDS development beyond anecdotal observations and practices. For example, during nursery production of daphne cultivars, many producers apply prophylactic fungicide treatments to help ensure crop health. While this is a relatively common practice, no published literature was found that directly addresses the efficacy of this practice to control DSDS. In addition, even if effective at controlling DSDS, reliance on fungicides is unsustainable and undesirable. One alternative to this practice is the development of disease resistant daphne cultivars. This strategy is typically more desirable because it can

be highly effective in reducing disease, is environmentally benign, and usually entails little or no additional expense to producers (Crute, 1996; Dahlberga, 2001; Diaz-Pérez 1995; Reeleder, 1999). However, it typically requires a long time horizon to achieve. To date, native host plant resistance to *T. basicola* has been identified during germplasm screens as part of various crop improvement or breeding programs. For example, *Nicotiana glauca* Graham was identified as resistant to *T. basicola* and subsequently incorporated into a tobacco (*N. tabacum*) breeding program (Trojak-Goluch, 2005). In another germplasm screen, *Gossypium arboreum* L. PI 1415 was found to be resistant to *T. basicola* and subsequently incorporated into a diploid cotton breeding program (Wheeler, 1999; Shankara, 1999).

Based on these data, I designed experiments to identify the casual agent of DSDS for subsequent use in a germplasm screen of *Daphne* species.

2.2 Material and Methods

2.2.1 Fungi isolation, inoculation and evaluation procedures

To identify the causal agent of DSDS, tissue samples were collected from diseased and healthy plants (paired samples of diseased and healthy plants acquired from individual nurseries throughout the greater Vancouver region) of *Daphne cneorum* 'Ruby Glow' which included roots of various diameters, discoloration, and degree of degradation. Special attention was paid to sampling tissue from the margins surrounding necrotic lesions. Samples were cut into 2-4 mm lengths and surface-disinfested with 10% bleach (5.25% sodium hypochlorite) for 5 min, and rinsed in water. These samples were

subsequently cultured on both general media (e.g., potato dextrose agar and corn meal agar) as well as media specific for an individual groups of fungi; e.g., cornmeal for detection of *Phytophthora* (Ferguson, 1999), Komada's medium for detection of *Fusarium* (Komada, 1975), V8 ® (Campbell Soup Co. Camden, NJ) for detection of *Phytophthora* or *Thielaviopsis basicola*, and carrot sections for specific detection of *Thielaviopsis basicola* (Tsao, 1970; Punja etal, 1992). After initial growth, all fungi were subsampled 3-5 times to eliminate bacterial contamination.

Following subculturing, single spore colonies were produced for each isolate. For each individual isolate, a spore suspension was prepared by gently washing the surface of 3-week-old colonies (abraded with a fine brush) with deionized water and vortexing the wash solution for 30 sec. The resulting suspension was twice filtered through four layers of cheesecloth to remove agar, hyphae, and other debris. The suspensions were calibrated with a haemocytometer and adjusted with deionized water to obtain a final concentration of $1x10^6$ ml^{-1} spores prior to inoculation. The concentration of spores chosen was based on preliminary experiments that determined the optimum concentration for effective inoculation (Appendix 1).

Following pure culture production and inoculum concentration determinations, isolate pathogenicities were tested by applying 5 ml of the conidial suspension to healthy roots of both 2-year old nursery-grown and rooted *in vitro*-produced plantlets of *D. cneorum*. Distilled water alone was applied to the control plants. Following inoculation, nursery-grown plants were kept under ambient conditions (24°C/14°C day/night averages over

the experimental period) while *in vitro* plantlets were maintained at 24 °C with a 16 h photoperiod. This temperature was chosen after observation of several isolates' growth rates under laboratory conditions. At this temperature, all isolates grew equally well with comparable vigor. The experiment was repeated over three different seasons of the year.

In these experiments, a completely randomized design was used with three replications. Each replication consisted of 15 plants (i.e., 45 plants for each fungal isolate) with two plants serving as controls. Descriptive statistics, analysis of variance, correlation, and Tukey's honest significant differences (HSD) test were generated using SPSS 11.5 software (SPSS Inc. Statistical Package for the Social Sciences, Chicago, US).

Weekly, data on disease progression were collected using the following 0-5 rating: 0= healthy plant, no symptoms; 1= less than five lesions on lateral roots, no lesions on tap root, no foliar symptoms; 2= greater than five lesions on lateral roots, less than five lesions on tap root, no foliar symptoms; 3= most lateral roots with lesions and some necrosis, greater than five lesions on tap root, five to ten chlorotic leaves; 4= most lateral roots necrotic, greater than five lesions on tap root, most leaves chlorotic with some leaf abscission; 5= plant is dead (Fig 2.1).

2.2.2 *Daphne* production

Rooted plants of *Daphne cneorum* were produced in July and August from terminal cuttings (50-100 mm in length) with the flower buds and lower leaves removed. Cuttings were made with a single shallow cut and soaked in an anti-fungal solution (Physan 20,

Maril Products Inc.) for 60 seconds. The cuttings were allowed to dry momentarily before being dipped in 0.4% IBA powder (Stim Root #2, Plant Products. Co. Ltd.) and then placed in 6 cm pots filled with a course rooting medium (10 parts propagation grade perlite, 8 parts peat, 6 parts granite grit #2, 1 part pumice (double screened to remove fine particles), dolomite lime 65AG at 900 g m^{-3}, and Micromax (trace elements; Scotts International Co., Geldermalsen, The Netherlands) at 400 g m^{-3}. The flats were placed under intermittent mist with bottom heat set at 22°C. Rooted cuttings were transferred to a polyhouse in October where they were allowed to go dormant but kept frost free. They were repotted in May into 12 cm pots filled with a well-drained medium (8 parts peat, 8 parts Turface MVP (Profile Products LLC, Buffalo Grove, IL 60089), 6 parts granite grit #2, 4 parts screened and pasteurized soil, 1 part pumice, dolomite lime 65AG at 670 g m^{-3}, Micromax micronutrients at 540 g m^{-3}, Osmocote (Scotts ® Miracle-Gro Co., Marysville, OH) 18-6-12 at 2150 g m^{-3}, and Psi Matric (TerraLink Horticulture Inc., Abbotsford, BC) wetting agent. All stock plants were grown under shade cloth during the summer months and moved to a heated polyhouse during the winter months to prevent frost damage. Fertilization regiment included yearly top-dressing with Osmocote 18-6-12 at 5 g l^{-1} gal pot. Fungicides were not used during stock production because very little disease pressure was present and I did not want to risk cross-contamination affecting the *in vivo* assay.

Prior to inoculations, nursery production containers were modified to contain a clear panel behind a lightproof 'door' to allow for direct observation of the infection process without further disturbance to the root system. All procedures were the same for control plants

except for the application of distilled water instead of the spore suspension. To allow for uniform conditions following inoculation, plants were transferred to a greenhouse and grown under natural light at 24 $\pm$1 °C and a relative humidity between 70 and 80%.

2.3 Results

From diseased plants, the following fungi were isolated: *Fusarium roseum* (Snyder & Hansen), *Fusarium oxysporum* (Snyder & Hansen), *Trichoderma* sp. (Persoon ex Gray), *Aspergillus* sp. (Micheli ex Link) and *Thielaviopsis basicola* (Berk. et Br.) Ferr. However, only *Thielaviopsis basicola* was isolated from all diseased plants but was absent from healthy plants. Identification was based on the presence of characteristic chlamydospores in root tissues. As mentioned earlier, *T. basicola* forms two spore types: hyaline cylindrical phialospores (endoconidia) and thick-walled pigmented chlamydospores (aleuriospores). As Nag Raj and Kendrick (1975) explain, both spore types are used as the basis for taxonomic identification of this species. Regardless of nursery- vs *in vitro*-based inoculations, all plants inoculated with *T. basicola,* developed stunted and chlorotic shoots with the roots displaying black lesions containing the characteristic spores of the fungus (Punja et al. 1992). These symptoms are consistent with those reported for DSDS. However, neither these symptoms, nor any other symptoms, were induced by any of the other fungi isolated from *Daphne* diseased tissue.

Comparing the nursery-inoculated plants to the *in vitro* inoculated plants four weeks post-inoculation, all nursery plants developed symptoms consistent with DSDS (average rating 3.5), while all other plants, either inoculated with the other isolates or clear water,

remained symptomless and healthy. In addition, *in vitro* inoculated plants displayed the same pattern of disease occurrence as expressed on the nursery-grown plants. However, *T. basicola* induced symptoms in significantly less time (< 2 weeks) on these plantlets than on the nursery-grown plants. Based on this observation, I conclude the temperature used during inoculation and disease evaluation (i.e., 24 C) demonstrates that this isolate of *T. basicola* can be an aggressive virulent pathogen to *Daphne* at this temperature. Furthermore, the higher temperature used during the *in vitro* evaluation, despite being higher than the optimum temperature reported for *T. basicola*, facilitated disease progression. The difference between my results and prior reports on temperature affects on *T. basicola* may be related to the fact that temperature guides for *T. basicola* are based on field, not laboratory, conditions. Field measured temperatures are typically heterogeneous with significant day/night fluctuations while laboratory conditions are significantly more consistent and stable. Following Koch's postulates, *T. basicola* was successfully re-isolated and re-identified from all plants expressing symptoms. Although my use of only one isolate of *T. basicola* (i.e., the most aggressive and robust isolate identified) imposes limitations on the breath of conclusions that can be made, (e.g., can not compare pathogenicity of other races on *Daphne*), I am able to conclude with confidence that *T. basicola* is the causal agent of Daphne Sudden Death Syndrome. This conclusion is based on the facts that only *T. basicola* was recovered from all diseased *Daphne* plants collected and that it was the only fungal isolate recovered able to induce DSDS.

2.4 References

Bottacin AM, Levesque CA and Punja ZK (1994) Characterization of dsRNA in *Chalara elegans* and effects on growth and virulence. Phytopathology. 84, 303 -312.

Crute IR and Pink DA (1996) Genetics and utilization of pathogen resistance in plants. The Plant Cell 8, 1747-1755.

Dahlberga JA and Bandyopadhyay R (2001) Evaluation of sorghum germplasm used in US breeding programmes for sources of sugary disease resistance. Plant Pathology. 50, 681-689.

Diaz-Pérez J (1995) Acclimatization and subsequent gas exchange, water relations, survival and growth of microcultured apple plantlets after transplanting them in soil. Physiologia Plantarum 95, 225-229.

Ferguson AJ (1999) Detecting multiple species of *Phytophthora* in container mixes from ornamental crop nurseries. Plant Disease. 83(12), 1129-1136.

Gayed SK (1972) Host range and persistence of *Thielaviopsis basicola* in tobacco soil. Can. J. Plant Sci. 52, 869–873.

Geldenhius M, Roux J, Wingfield M and Wingfield B (2004) Development of polymorphic markers for the root pathogen Thielaviopsis basicola using ISSR-PCR. Mol. Eco. Notes. 4, 547–550.

Grand LF (1985) North Carolina Plant Disease Index. North Carolina Agri. Res. Ser. Tech. Bul. 240, 1-157.

Hood ME and Shew HD (1996) Pathogenesis of *Thielaviopsis basicola* on a susceptible and a resistant cultivar of burley tobacco. Phytopathology. 86, 38-44.

Hood ME and Shew HD (1997a) Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs. Phytopathology. 87, 228-235.

Hood ME and Shew HD (1997b) Reassessment of the role of saprophyti activity in the ecology of *Thielaviopsis basicola*. Phytopathology. 87, 1214-1219.

Howell C (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Disease. 87, 4-10.

Komada H (1975) Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soils. Rev. Plant Prot. Res. 8, 114-124.

Linderman RG and Zeitoun F (1977) *Phytophthora* causing root rot and wilt of nursery-grown native western azalea and other ornamental plants. Plant Dis. Rep. 16(12), 2-3.

Mauk PA and Hine RB (1988) Infection, colonization of *Gossypium hirsuturn* and *G. barbadense* and development of black root rot caused by *Thielaviopsis basicola.* Phytopathology. 78, 1662-1667.

Nag Raj TR and Kendrick B (1975) A monograph of *Chalara* and allied genera. Wilfred Laurier University Press, Waterloo, Ont.

Pany D (1990) Plant Pathology in Agriculture. Cambridge University Press, Cambridge.

Park Y, James D and Punja ZK (2005) Co-infection by two distinct totivirus-like double-stranded RNA elements in *Chalara elegans* (*Thielaviopsis basicola*). Virus Research. 109, 71–85.

Pataky NR (1988) Report on Plant Disease. RPD No. 650, Department of Crop Sciences, University of Illinois at Urbana-Champaign.

Punja ZK Chittaranjan S and Gaye MM (1992) Development of black root rot caused by *Chalara elegans* on fresh market carrots. Can. J. Plant Pathology. 14, 299–309.

Punja ZK (1993) Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*. Can. J. Bot. 71, 447-456.

Punja ZK (1995) Influence of double-stranded RNAs on growth, sporulation, pathogenicity, and sumival of *Chalara elegans*. Can. J. Bot. 73, 1001-1009.

Reeleder R (1999) Septoria leaf spot of *Stevia rebaudiana* in Canada and methods for screening for resistance. J. of Phytopathology. 147, 605-613.

Rothrock CS (1992) Influence of soil temperature, water, and texture on *Thielaviopsis basicola* and black root rot of cotton. Phytopathology.82, 1202-1206.

Stover RH (1950a) The black root-rot disease of tobacco. I. Studies on the causal organism *Thielaviopsis basicola*. Can. J. Res. Sect. C Bot. Sci. 28, 445–470.

Stover RH (1950b) The black root-rot disease of tobacco. II. Physiologic specialization of *Thielaviopsis basicola* on *Nicotiana tabacum*. Can. J. Res. Sect. C Bot. Sci. 28, 726–738.

Tompkins CM (1951) Stem and root rot of *Daphne odora* caused by *Phytophthora parasitica*. Phytopathology. 41, 654-656.

Trojak-Goluch A and Berbec A (2007) Meiosis and fertility in interspecific hybrids of *Nicotiana tabacum* L., *N. glauca* Grah. and their derivatives. Plant Breeding 126, 201-206.

Troxler J and berling C (1997) Interactions between the biocontrol agent *Pseudomonas fluorescens* CHA0 and *Thielaviopsis basicola* in tobacco roots observed by immunofluorescence microscopy. Plant Pathology. 46, 62-71.

Tsao PH (1970) Selective media for isolation of pathogenic fungi. Ann. Rev. Phytopathology. 8, 157-186.

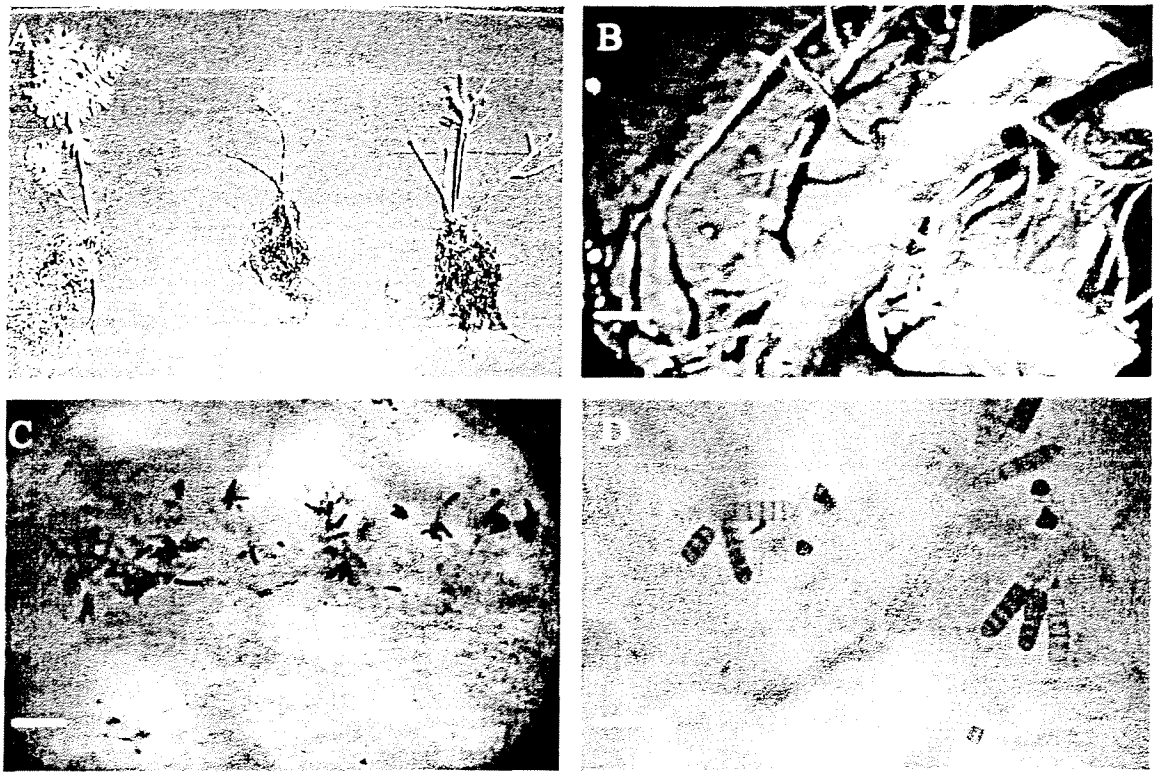
Walker N, Kirkpatrick T, and Rothrock C (1999) Effect of temperature on and histopathology of the interaction between *Meloidogyne incognita* and *Thielaviopsis basicola* on cotton. Phytopathology. 89 (8) 613-617.

Wattimena S (2001) Studies of factors affecting virulence of *Chalara Elegans* on Bean (Phaseolus Vulgaris L.). MSc. Thesis. Simon Fraser University.

Wheeler TA, Gannaway JR and Keating K (1999) Identification of resistance to *Thielaviopsis basicola* in diploid cotton. Plant Disease. 83, 831-833.

Yarwood CE (1981) Occurrence of *Chalara elegans*. Mycology. 73, 524-530.

Figure 2.1. Infection of *D. cneorum* 'Ruby Glow' by *Thielavopsis basicola*: (A) Stages of disease progression from left to right- healthy shoot (rating of 0), lesions on fine roots; leaf abscission, fine root necrosis (rating of 4); plant death (rating of 5); (B) Roots showing lesions and tissue necrosis, (circle indicates typical region for sample collection), scale bar= 0.33 mm; (C) *T. basicola* chlamydospores in root tissue, scale bar= 50µm; (D) *T. basicola* chlamydospores in culture, scale bar= 25µm.



CHAPTER THREE [1]

IN VITRO PROPAGATION OF SEVEN *DAPHNE L.* SPECIES

3.1 INTRODUCTION

The genus *Daphne* L. (Thymelaeaceae) is comprised of 95 recognized species (Flora of China, 2005) distributed primarily in Europe and Asia with a few species endemic to Africa and Australia. This genus of flowering plants includes deciduous, semi-evergreen and evergreen shrubs. Many species are spring flowering and have deliciously fragrant flowers that range in color from white to purple with a few having greenish-yellow or yellow flowers. Plant habits range from low growing prostrate forms, only a few inches high, to large shrubs. Several species have been commercially propagated because of their desirable horticultural characteristics, including attractive foliage, plant habit, flower color and most of all, pleasant fragrance. Specifically, *D. cneroum* L. (rose daphne or garland flower) has become one of the most popular perennial flowering shrubs among discriminating ornamental plant growers (Brickell and White, 2000a; Halda, 2001). Commercial propagation of *Daphne* is typically from seed or vegetative cutting. However, seeds are genetically variable adding undesirable variation to a production system.

---

[1] A version of this chapter has been submitted for publication.

Noshad D, Riseman A. In vitro propagation of Seven *Daphne L.* Species

Vegetative propagation is more desirable but root induction is very difficult for many *Daphne* taxa (Brickell and White, 2000a; Mathew et al, 2000). In addition to rooting difficulties, commercial production of some species was in decline due to the build-up of internal pathogens within the stock plants (Noshad, 2006; Green etal, 1992; Hartmann and Preece, 1990; Havel and Kolar, 1983). However, these species were successfully 'refreshed' by producing new stock plants through tissue culture techniques, thereby eliminating most of the internal pathogens (Jayasankar etal, 2001; Chen etal, 1995). Although *in vitro* techniques successfully generated disease-free plants in a relatively short time period (Bajaj, 1996), *Daphne* species remain very difficult to establish and manipulate in culture. Part of the problem appears to be associated with significant species-specific culture requirements and the presence of polyphenol oxidases in *Daphne* tissue that cause explant browning and death during establishment (Marks and Simpson, 2000; Green etal, 1992; Cohen, 1977). Therefore, the development of species-specific *in vitro* protocols is needed and will allow for the production of uniform, disease-free plants for commercial and experimental use (Krogstrup and Norgaard, 2001; Bennett and Davis, 1986). Rooting and in vivo establishment of micropropagated *Daphne* has also proven difficult for several species (Tricoli etal, 1985). Therefore, the objective of this study was to develop efficient establishment, proliferation, and rooting protocols for the *in vitro* propagation of selected *Daphne* species. The present work established protocols for the micropropagation of seven species of *Daphne*.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growing conditions

Seven *Daphne* species, collected by the UBC Botanical Garden, Vancouver, BC, Canada, were used in this research and included *D. cneorum* L., *D. caucasica* Pall., *D. retusa* Hemsl., *D. giraldii* Nitsche, *D. jasminea* Sibth.& Sm., *D. laureola* L. and *D. tangutica* Maxim. Apical shoot tips, 2-5 cm in length, 3-5 mm in diameter, and bearing 1-3 nodes were collected during the summer and fall seasons from 4-6 year old container-grown plants. Collected shoots were stripped of all leaves and rinsed under running tap water for 15 min. Under aseptic conditions, shoots were then surface sanitized by treatment with 70% ETOH for 30 seconds followed by treatment with 0.5% sodium hypochlorite solution containing 0.5 ml l^{-1} Tween-20 (Sigma Chemical, St. Louis, MO) and gently stirred for 10 min. After sanitation, shoots were rinsed three times with sterile distilled water for 5 min each and placed individually in culture vessels. Cultures were maintained at 25°C under 16 h photoperiod with irradiance intensity of 350 $\mu W\ cm^{-2}$ supplied by cool white fluorescent lamps. Following this disinfestations procedure, culture contamination rates were below 10%.

3.2.2 Establishment phase

Individual nodal explants 1-2 cm long were cut aseptically and positioned vertically in individual 25 x 150 mm test tubes containing 25 ml of medium. Several base media were evaluated and included MS (Murashige and Skoog, 1962), WPM (Woody Plant Medium; McCown and Lloyd, 1983), B5 (Gamborg etal, 1968), LS (Linsmaier & Skoog, 1965), and SH (Schenk and Hildebrandt, 1972). All base media were supplemented with 20 g l^{-1} sucrose,

and 5.6 g l^{-1} high gel strength agar (Sigma-Aldrich, St. Louis, MO). The pH of the various media was adjusted based on published protocols (e.g., MS media was adjusted to 5.8, WPM media to 5.2) before autoclaving for 15 min at 121°C. Experiments designed to determine the effects of autoclaving on PGR activities found no significant reduction in activity following these sterilization procedures (Appendix 2). Explants were maintained on establishment media for four weeks prior to subculture on multiplication media.

3.2.3 Multiplication phase

Following a four week establishment period, shoots were sub-cultured into 150 mm baby food jars (Sigma-Aldrich, St. Louis, MO) containing 25 ml multiplication media. Shoots were harvested from stock cultures, cut into nodal explants 3-5 cm long and place vertically into media containing either MS or WPM basal salts, and 5.6 g l^{-1} agar (Sigma-Aldrich, St. Louis, MO). Based on explant growth during the establishment phase, *D. cneorum* and *D. jasminea* were sub-cultured on WPM based media while the other species were sub-cultured on MS based media (Table 3.1). Various concentrations of 6- benzyl aminopurine (BA), kinetin (KIN), and thidiazuron (TDZ) were used alone or in conjunction with one of three concentrations of indole-3 acetic acid (IAA), indole 3- butyric acid (IBA), or α-naphthalene acetic acid (NAA) (Tables 3.2 and 3.3). The experiment was conducted in a randomized complete block design with three replications. Shoot proliferation data were based on the number of 'usable' shoots (>1 mm long) produced per explant after 8 wks in culture (Table 3.2).

3.2.4 Elongation and rooting phases

Following multiplication, new shoots were used in elongation and rooting trials. For shoot elongation, shoots 1 cm long with intact apices and 2-4 leaves were cultured on either MS or WPM basal medium supplemented with 1 g l^{-1} charcoal. After four weeks, the longest shoots (4-6 cm) were transferred to individual test tubes with 20 ml rooting media consisting of standard MS or WPM salts, vitamins (nicotinic acid 0.5 g l^{-1}, pyridoxine-HCl 0.5 g l^{-1}, thiamine-HCl 1 g l^{-1}, and glycine 2 g l^{-1}), 20 g l^{-1} sucrose, and 5.6 g l^{-1} agar. In addition, shoots were exposed to various combinations of PGRs through one of three methods as follows (individual PGR treatments used are detailed in Tables 3.4, 3.5 and 3.6):

1) *conventional method*: PGRs were added to the medium before autoclaving and were homogeneous within the container (Table 3.4);

2) *two-layer method*: two media layered with the upper basal medium containing PGR(s) and the lower basal medium with charcoal but without PGR(s) (Table 3.5);

3) *PGR dip method*: shoots were directly dipped into a PGR solution before culture on basal medium sans PGR(s). For this method, all PGRs were filter sterilized before use. Shoot bases (lower 2-3 cm) were dipped for 5, 15, or 30 mins in 1 mM solution of an individual PGR or dipped for 15 min in a combination of PGRs (Table 3.6). Shoots were then transferred directly to basal medium without PGRs. Rooting, expressed as the percentage of shoots producing root initials >5 mm in length, was recorded after 8 weeks. Preliminary experiments were conducted to evaluate the effects of changes in component concentrations of the base media (Appendix 3). I found all alterations to the published media compositions tested inferior for *Daphne* rooting.

3.2.5 Experimental design and analyses

All experiments used a complete randomized design with three replications and 15 shoots per replication (i.e. 45 shoots). Data were recorded as number of multiplied shoots after 8 weeks, and percentage of shoots rooted after another 8 weeks. The percentage data were subjected to arcsin transformation before ANOVA analysis. Descriptive statistics, analysis of variance, Tukey's honest significant differences (HSD) test, and other analysis results were generated using SPSS® 11.5 software (SPSS Inc. Statistical Package for the Social Sciences, Chicago, US).

3.3 RESULTS

3.3.1 Establishment phase

All primary explants harvested from stock plants were contamination-free 2 weeks after establishment. In addition, the inclusion of activated charcoal was effective in reducing the negative effects of phenolic compounds produced by these initial explants. Significant medium effects were observed for species survivorship after two weeks with MS-based media most appropriate for five species (*D. caucasica*, *D. giraldii*, *D. laureola*, *D. retusa*, and *D. tangutica*) and WPM-based media most appropriate for the remaining two species (*D. cneorum* and *D. jasminea*) (Table 3.1). The remaining three media were ineffective in supporting growth of any *Daphne* species evaluated. Axillary meristems from all species initiated growth after 2 weeks of culture in a species-most-favorable medium (Table 3.1) and grew to 10-15 mm after 4 weeks.

3.3.2 Multiplication phase

Significant effects were observed for axillary shoot production by species and media (Tables 3.2 and 3.3; Fig 3.1). Continuing to follow the medium preference observed earlier, *D. caucasica, D. giraldii, D. retusa, D. laureola* and *D. tangutica* displayed the greatest proliferation on MS-based media, while *D. jasminea* and *D. cneorum* displayed the greatest proliferation on WPM-based media. When apical and lateral explants were compared, no significant differences in multiplication capacity were detected.

In addition to base media effects, significant effects on multiplication rate were observed for PGR supplement. When neither cytokinin nor auxin were incorporated into media, shoots grew with strong apical dominance without axillary shoot proliferation. When a cytokinin was incorporated without auxin, the BA treatments produced the greatest multiplication rates with increasing response observed with increasing concentrations up to 2 mg l^{-1} (Table 3.2). With the incorporation of one cytokinin, *D. retusa* produced the greatest proliferation rate with an average of 1.56±0.5 shoots per explant. KIN treatments also induced greater response with increasing concentrations, but the overall rates remained below those of BA. Shoots induced on BA-containing media were usually of normal appearance and, in the moderate concentration ranges, did not cause any hyperhydricity. The TDZ treatments were less effective in higher concentrations than the other two cytokinins tested with a general trend of decreasing response as concentration increased. In addition, TDZ induced a very high frequency of hyperhydricity.

When a cytokinin was used in combination with an auxin, increases in multiplication rates were observed. Incorporation of NAA, regardless of cytokinin or species, greatly increased multiplication rates over those of IAA, IBA or the use of the cytokinin alone (Table 3.3). Among species, *D. tangutica* produced the greatest proliferation rate with an average of 3.6±0.2 shoots per explant (Table 3.2). *D. caucasica, D. giraldii*, and *D. laureola* produced the greatest number of shoots per explant when cultured on medium supplemented with 2 mg l^{-1} BA+ 0.01 mg l^{-1} NAA while *D. retusa* and *D. tangutica* responded best to medium supplemented with 1 mg l^{-1} BA + 0.01 mg l^{-1} NAA. However, the remaining two species, *D. cneorum* and *D. jasminea*, produced their greatest respective multiplication rates when two cytokinins (0.5 mg l^{-1} BA and 0.001 mg l^{-1} TDZ) were combined with 0.01 mg l^{-1} NAA (Table 3). In general, BA was more efficient for inducing shoot formation than other cytokinins. Also, combinations of cytokinins and auxin resulted in a higher number of shoots with the combination of BA and NAA inducing very strong shoot proliferation (Table 3.3).

3.3.3 Rooting and acclimation

Root initiation and development required approximately 60 days (Fig 3.2) with significant treatment effects on the frequency of rooting observed (Table 3.4, 5 and 6). Root development was induced by the addition of auxin, either NAA and/or IBA, for all species tested with variable rates. No rooting occurred in the absence of auxins. Generally, the inclusion of IBA or NAA resulted in a higher number of roots than IAA. Treatments with only NAA resulted in the formation of thicker roots than roots induced by IBA. The two-layer media and dipping techniques were superior in inducing rooting than the traditional medium with homogeneous PGR incorporation (Table 3.5 and 6). Regardless of species,

both techniques produced rooting frequencies of >85% in some individual PGR combinations (Table 3.5 and 6). However, explants in the two-layer media often produced callus and/or adventitious roots from callus, while no such growth was observed with the dipping method. Therefore, the dipping technique appeared to be the most efficient technique for use with these selected species (Table 3.6). Rooted shoots were successfully acclimatized in a fog chamber and survived transfer to the greenhouse at >85%.

3.4 DISCUSSION

Daphne has been categorized as a "difficult-to-root" group, both with conventional propagation via cuttings and following micropropagation (Brickell and white, 2000b; Marks and Simpson, 2000). In addition to rooting difficulties, browning of tissue and hyperhydricity (or vitrification) are reported to be problematic during *in vitro* propagation of several *Daphne* species (Chen and Li, 1995; Wang and Tang, 1994; Cohen, 1977). My research is the first systematic attempt to optimize the protocols for *in vitro* propagation and root induction of a number of *Daphne* species. My results mostly support previous research on the micropropagation of one species, *D. cneorum* (Mala etal, 2004; Marks and Simpsons, 2000) having produced similar results. However, new optimizations of these protocols are now established as well as the addition of species-specific protocols for an additional six species.

In general, species responded differently to media, individual PGRs, combinations of PGRs, rooting technique, and required species-specific protocol development. With species-specific protocols optimized, successful multiplication was achieved without the common problems of

browning or hyperhydricity. Adding activated charcoal to the media was very helpful for the control of browning in all species. Hyperhydricity was observed more often in some treatments than in others. Reports suggest hyperhydricity could result from the type and concentration of cytokinins, gelling agent, or medium used (Preece and Compton, 1991; Escobar and Villalobos, 1986; Krulik, 1980). My cultures usually became vitrified on media with high concentrations of cytokinins, particularly with TDZ. High TDZ concentrations have been shown to induce hyperhydricity in several other plant species e.g. *Populus tremula* (Vinocur et al., 2000; Clayton and Hubstenberger, 1995; Huetteman and Preece, 1993; Garton etal, 1981) and support my findings. This cytokinin-like compound has been used for microprogation of many woody species (Zhang et al., 2001; Clayton and Hubstenberger, 1995; Mohamed-Yasseen etal, 1994; Drew etal, 1993), but has not been widely tested for members of the genus *Daphne*. Based on TDZ concentrations comparable to those used by other authors for shoot induction (Mulwa and Bhalla, 2000; Martinez-Vazquez and Rubluo, 1989; McComb, 1985; Tricoli etal, 1985), I do not recommend TDZ use in *Daphne* micropropagation. This investigation also revealed that auxin supplementation was required for *in vitro* rooting. However, species still responded slowly (approximately 7-9 weeks) to the various rooting treatments.

3.7 REFERENCES

Bajaj YPS (1996) Biotechnology in Agriculture and Forestry Trees IV. 35, 20–290. Springer-Verlag, Berlin.

Bennett CK and Davis J (1986) *In vitro* propagation of *Quercus schumerida* seedling. HortSci. 21, 1045–1047.

Brickell CD and White R (2000*a*) A quartet of New Daphnes. The New Plantsman. March, 6-18. The Royal Horticultural Society.

Brickell CD and White R (2000*b*) Further trio of new Daphne hybrids. The New Plantsman. December, 236-248. The Royal Horticultural Society.

Chen DZ, Liu T and Li Y (1995) Studies on rapid clonal propagation of *Daphne odora in vitro* culture. Acta Hort. 404, 15-20.

Clayton PW and Hubstenberger JF (1990) Micropropagation of members of the Cactaceae Subtribe Cactinae. J. Am. Soc. Hort. Sci. 115, 337–343.

Cohen D (1977) Thermotherapy and meristem-tip culture of some ornamental plants. Acta Hort. 78, 381-388.

Drew RA, McComb JA and Considine JA (1993) Rhizogenesis and root growth of *Carica papaya* L. *in vitro* in relation to auxin sensitive phases and use of riboflavin. Plant Cell Tiss. Org. Cult. 33, 1–7

Escobar H and Villalobos V (1986) Opuntia micropropagation by axillary proliferation. Plant Cell Tiss. Org. Cult. 7, 269–277.

Flora of China Vol. 13 (*Clusiaceae through Araliaceae*). (2005) Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Gamborg, OL, Miller, RA and Ojima, K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 50, 151–158

Garton S, Hosier MA and Read PE (1981) *In vitro* propagation of *Alnus glutinosa* Gaertn. Hort Sci. 16, 758-759

Green MJ, Godkin SE and Monette PL (1992) Use of *in vitro* cultures of *Daphne cneorum* L. for the western detection of daphne virus X. J. Enviro. Hort. 10(3), 153-155.

Halda JJ (2001) The Genus *Daphne*. Eva Kucerova- Sen Dobre Publikaca, Prague, Czeck Republic.

Hartmann HT and Kester DE (1990) Plant propagation: principles and practices. Englewood Cliffs, NJ: Prentice Hall.

Havel L and Kolar Z (1983) Microexplant isolation from Cactaceae. Plant Cell Tiss. Org. Cult. 2, 349–353.

Huetteman CA and Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33, 105–119.

Jayasankar S, Vanaman M, Li Z and Gray DJ (2001) Direct seeding of grapevine somatic embryos and regeneration of plants. In Vitro Cell & Dev. Biol. - Plant 37: 476-479(4)

Krogstrup P and Norgaard J V (1991) Micropropagation of *Psiadia coronopus* (Lam.) Benth, a threatened endemic species from the island of Rodrigues. Plant Cell, Tiss. Org. Cult. 27, 227-230.

Krulik G (1980) Tissue culture of succulent plants. Nat. Cact. Succ. J. 35,14–17.

Linsmaier E and Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100–127.

Mala J and V. Bylinský (2004) Micropropagation of endangered species *Daphne cneorum*. Biol. Plant. 48 (4), 633-636.

Marks T and Simpson S (2000) Interaction of explant type and indol-3-butyric acid during rooting *in vitro* in a range of difficult and easy to root woody plants. Plant Cell, Tiss. Org. Cult. 62, 65-74.

Martinez-Vazquez O and Rubluo A (1989) *In vitro* mass propagation of the near-extinct *Mammillaria sanangelensis* Sanchez-Mejorada. J. Hort. Sci. 64, 99–105.

Mathew B, Brickell C and White R (2000) The smaller Daphnes. The Proceeding of the Conference " Daphne 2000 " April 2000. The Royal Horticultural Society, London.

McComb JA (1985) Micropropagation of the rare species *Stylidium coroniforme* and other *Stylidium* species. Plant Cell Tiss. Org. Cult. 4, 151–158.

McCown BH and Lloyd GB (1983) A survey of the response of *Rhododendron* to *in vitro* culture. Plant Cell Tiss. Org. Cult. 2, 75–85

Mohamed-Yasseen Y (1994) Micropropagation of pitaya (*Hylocereus undatus* Britt. et Rose). HortSci. 29, 559-560.

Mulwa RMS and Bhalla PL (2000) *In vitro* shoot multiplication of *Macadamia tetraphylla* L. Johnson. J. Hortic. Sci. Biotechnol. 75, 1–5.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Noshad D, Punja ZK and Riseman A (2006) First report of *Thielaviopsis basicola* on *Daphne cneorum*. Can. J. Plant Pathol, 28(3), 310-312.

Preece JE and Compton ME (1991) Problems with explant exudation in micropropagation. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, 17, 168–169. High-tech and Micropropagation 1. Springer-Verlag, Berlin.

Schenk RU and Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199–204.

Tricoli DM, Maynard CA and Drew AP (1985) Tissue culture propagation of mature trees of *Prunus seritona* Ehrh. I. Establishment, multiplication and rooting of *in vitro*. For. Sci. 31, 201–208.

Vinocur B, Carmi T, Altman A and Ziv M (2000) Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media. Plant Cell Rep.19, 1146–1154.

Wang Q and Tang H (1994) Phenol induced browning and establishment of shoot-tip explants of fuji apple and jinhua pear cultured *in vitro*. J. Hort. Sci. 69, 833-839.

Zhang CH, Chen D, Elliott MC and Slater A (2001) Thiazuron-induced organogenesis and somatic embryogenesis in sugar beet (*Beta vulgaris* L.). In Vitro Cell. Dev. Biol.—Plant 37, 305–310.

Table 3.1. Explant survivorship (%) among *Daphne* species following a 4 wk establishment phase on one of five basal media without PGR supplements; mean percent ± SD.

Species	MS	WPM	B5	SH	LS
D. caucasica	82±2.1	2.5±1.5	0	2.5±1.6	0
D. cneorum	5±0.3	85±3.8	0	0	0
D. giraldii	87±3.8	5±2.4	5±1.3	0	0
D. jasminea	7±1.6	92±2.1	0	0	0
D. laureola	75±0.8	2.5±0.6	0	0	0
D. retusa	92±3.8	2.5±0.8	0	0	0
D. tangutica	92±2.6	5±1.3	0	0	2.5±1.1

Table 3.2. Shoot explant multiplication rates following 8 weeks of culture on basal media supplemented with a single cytokinin and no auxin; mean ± SD.

Treatment	**Species**						
Cytokinin Supplement	***D. caucasica***	***D. cneorum***	***D. giraldii***	***D. jasminea***	***D. laureola***	***D. retusa***	***D. tangutica***
No cytokinin	0.33±0.0	0.06±0.4	0.13±0.0	0.20±0.3	0.20±0.0	0.46±0.2	0.48±0.1
0.1 mg l^{-1} BA	0.80±0.1	0.08±0.3	0.31±0.1	0.20±0.4	0.26±0.1	1.02±0.8	0.93±0.3
0.5 mg l^{-1} BA	1.51±0.3	0.28+0.2	1.75±0.3	0.44±0.0	0.82±0.5	2.22±1.2	2.08±0.8
1 mg l^{-1} BA	1.77±0.5	0.53±0.1	2.04±1.1	0.60±0.3	1.64±1.1	2.13±0.8	2.02±0.5
2 mg l^{-1} BA	2.46±1.1	0.66±0.3	2.71±0.8	0.73±0.2	1.95±0.8	2.55±1.4	2.60±1.3
4 mg l^{-1} BA	1.82±0.3	0.33±0.2	2.28±1.3	0.40±0.1	1.77±1.5	2.53±0.3	2.33±0.9
0.1 mg l^{-1} KIN	0.46±0.2	0.06±0.1	0.46±0.6	0.13±0.3	0.20±0.1	0.53±0.1	0.46±0.1
0.5 mg l^{-1} KIN	0.62±0.3	0.06±0.0	0.55±0.3	0.20±0.1	0.60±0.4	0.80±0.6	0.60±0.5
1 mg l^{-1} KIN	1.22±0.8	0.20±0.4	1.66±1.1	0.40±0.6	0.86±0.3	1.13±0.7	1.17±0.8
2 mg l^{-1} KIN	1.73±0.0	0.28±0.3	1.40±0.8	0.42±0.3	1.77±0.5	2.20±1.1	1.71±0.7
4 mg l^{-1} KIN	1.82±0.3	0.20±0.1	1.42±0.5	0.33±0.2	1.68±1.2	1.97±0.3	1.86±1.1
0.0001 mg l^{-1}TDZ	0.91±0.5	0.13±0.0	0.73±0.3	0.26±0.1	0.86±0.6	1.33±0.2	1.26±0.0
0.001 mg l^{-1}TDZ	1.91±0.3	0.42±0.1	1.33±0.7	0.73±0.5	1.66±0.9	1.55±1.1	1.57±0.2
0.01 mg l^{-1}TDZ	1.33±0.7	0.42±0.2	1.22±0.5	0.66±0.3	1.73±0.5	1.88±1.2	1.53±0.5
0.1 mg l^{-1}TDZ	1.06±0.8	0.22±0.3	1.08±0.8	0.33±0.2	1.42±1.1	1.86±0.8	0.95±0.6
0.2 mg l^{-1}TDZ	0.84±0.1	0.13±0.2	0.86±0.3	0.06±0.1	0.55±0.8	0.86±0.5	0.93±0.8
Average	**1.28±0.3**	**0.25±0.1**	**1.24±0.4**	**0.38±0.1**	**1.12±0.3**	**1.56±0.5**	**1.4±0.4**

Table 3.3. Shoot explant multiplication rates following 8 weeks of culture on basal media supplemented with a combination of cytokinins and auxins; ($P< 0.05$).

Supplement	Species						
	D. caucasica	*D. cneorum*	*D. giraldii*	*D. jasminea*	*D. laureola*	*D. retusa*	*D. tangutica*
1 mg l^{-1} BA + 0.001 mg l^{-1} IAA	1.9±0.3	1.46±0.6	1.80±0.8	1.55±0.3	1.46±0.5	1.93±0.4	2.13±0.1
1 mg l^{-1} BA + 0.01 mg l^{-1} IAA	1.9±0.9	2.13±0.0	2.42±0.3	2.40±0.0	2.08±0.3	2.26±0.2	2.33±0.0
1 mg l^{-1} BA + 0.1 mg l^{-1} IAA	2.2±0.6	2.06±0.2	2.15±0.3	2.00±0.0	2.15±0.3	2.35±0.0	2.40±0.3
2 mg l^{-1} BA + 0.001 mg l^{-1} IAA	1.6±0.5	1.71±0.3	1.93±0.1	2.13±0.6	1.33±0.0	2.06±0.4	2.00±0.2
2 mg l^{-1} BA + 0.01 mg l^{-1} IAA	1.91±0.0	2.13±0.8	2.75±0.2	2.26±0.2	2.26±0.4	2.86±0.7	2.84±0.2
2 mg l^{-1} BA + 0.1 mg l^{-1} IAA	2.20±0.3	2.26±0.7	2.66±0.4	1.93±0.5	2.53±0.6	2.75±0.3	2.80±0.2
1 mg l^{-1} BA + 0.001 mg l^{-1} NAA	4.00±0.8	3.20±0.9	4.33±0.3	5.35±0.3	4.06±0.1	5.31±0.3	5.35±0.4
1 mg l^{-1} BA + 0.01 mg l^{-1} NAA	5.33±0.7	5.06±0.6	4.91±0.8	6.93±0.7	5.17±0.8	6.48±0.2	7.73±0.8
1 mg l^{-1} BA + 0.1 mg l^{-1} NAA	3.73±0.8	4.57±0.8	4.37±0.2	5.28±0.3	3.64±0.5	4.60±0.5	4.48±0.3
2 mg l^{-1} BA + 0.001 mg l^{-1} NAA	4.66±0.5	3.40±0.0	4.64±0.3	6.13±0.9	4.80±0.2	5.00±0.6	5.13±0.0
2 mg l^{-1} BA + 0.01 mg l^{-1} NAA	5.64±0.7	5.13±0.4	5.06±1.1	6.46±0.2	5.46±0.5	5.46±0.6	5.66±0.0
2 mg l^{-1} BA + 0.1 mg l^{-1} NAA	4.31±0.8	4.66±0.3	4.77±0.3	5.53±0.2	4.73±0.4	4.66±0.9	4.55±0.3
1 mg l^{-1} BA + 0.001 mg l^{-1} IBA	2.75±0.0	2.60±0.9	2.93±0.0	3.66±0.3	2.75±0.4	3.08±0.4	3.13±0.0
1 mg l^{-1} BA + 0.01 mg l^{-1} IBA	3.71±0.9	3.04±0.3	3.64±0.3	3.91±0.4	3.91±0.8	4.06±0.5	4.28±0.3
1 mg l^{-1} BA + 0.1 mg l^{-1} IBA	3.06±0.6	2.75±0.7	3.40±0.1	3.20±0.6	3.46±0.2	3.97±0.3	3.93±0.0
2 mg l^{-1} BA + 0.001 mg l^{-1} IBA	2.93±0.7	2.66±0.0	2.86±0.6	3.57±0.3	2.97±0.6	3.00±0.1	3.06±0.0
2 mg l^{-1} BA + 0.01 mg l^{-1} IBA	3.93±0.9	3.13±0.1	3.66±0.2	3.84±0.3	4.06±0.9	4.15±0.3	4.24±0.3
2 mg l^{-1} BA + 0.1 mg l^{-1} IBA	3.37±0.6	2.86±0.7	3.26±0.0	3.80±0.0	3.86±0.4	4.22±0.3	3.84±0.3
2 mg l^{-1} KIN + 0.01 mg l^{-1} IAA	1.95±0.3	1.33±0.0	2.84±0.3	1.68±0.3	1.88±0.2	2.55±0.2	2.40±0.0
2 mg l^{-1} KIN + 0.01 mg l^{-1} NAA	3.06±0.4	2.73±0.8	3.06±0.1	2.57±0.3	2.93±0.2	2.64±0.2	3.13±0.0
2 mg l^{-1} KIN + 0.01 mg l^{-1} IBA	2.24±0.2	1.77±0.3	2.75±0.3	1.93±0.0	2.28±0.4	2.66±0.1	2.86±0.0
4 mg l^{-1} KIN + 0.01 mg l^{-1} IAA	1.86±0.5	2.17±0.2	2.20±0.5	2.13±0.4	1.93±0.3	2.13±0.0	2.08±0.3
4 mg l^{-1} KIN + 0.01 mg l^{-1} IAA	2.66±0.8	2.60±0.4	2.64±0.3	2.60±0.4	2.73±0.3	2.95±0.3	3.04±0.3
4 mg l^{-1} KIN + 0.01 mg l^{-1} IAA	2.08±0.7	2.35±0.3	2.40±0.4	2.40±0.3	2.15±0.7	2.53±0.0	2.33±0.2
0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} IAA	2.84±0.9	1.86±0.2	2.06±0.6	2.33±0.5	2.91±0.4	2.93±0.2	3.00±0.3
0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} NAA	3.57±0.5	2.62±0.3	2.75±0.3	2.86±0.7	3.71±0.5	3.20±0.6	3.26±0.4
0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	3.13±0.2	2.33±0.0	2.91±0.7	2.55±0.1	3.17±0.3	3.00±0.2	2.93±0.1
0.001 mg l^{-1} TDZ + 0.1 mg l^{-1} IAA	2.11±0.8	2.17±0.3	2.26±0.4	2.26±0.4	2.28±0.2	2.55±05	2.13±0.3
0.001 mg l^{-1} TDZ + 0.1 mg l^{-1} NAA	2.91±0.9	2.42±0.3	2.40±0.2	2.55±0.2	3.13±0.4	3.00±0.7	2.86±0.0
0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	2.55±0.2	2.08±0.3	2.33±0.7	2.33±0.3	2.86±0.0	2.53±0.4	2.51±0.7
0.5 mgl^{-1} BA+0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} IAA	4.93±0.8	4.60±0.0	4.13±0.9	4.82±0.6	3.80±0.3	4.28±0.4	4.84±0.3
0.5 mgl^{-1} BA+0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	4.53±0.7	4.35±0.3	4.15±0.4	4.46±0.8	3.64±0.7	4.68±0.8	4.42±0.1
0.5 mgl^{-1} BA+0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} NAA	5.57±0.7	5.37±0.3	4.33±0.5	7.84±0.6	4.04±0.8	5.46±1.1	6.86±0.8
0.5 mgl^{-1} BA+0.01 mg l^{-1} TDZ + 0.01 mg l^{-1} NAA	5.26±0.6	5.26±0.6	4.40±0.6	5.46±0.9	4.15±0.7	4.88±0.9	5.13±0.6
Average	**3.24±0.6**	**2.96±0.3**	**3.2±0.4**	**3.55±0.5**	**3.18±0.4**	**3.5±0.3**	**3.6±0.2**

F(49, 100)= (P< 0.05)	408.9	1045.7	4137.7	2871.2	6528.5	7788.9	9755.5

Table3.4. *Daphne* rooting frequency (%) after 8 weeks of culture on media incorporating PGRs

Supplement/Modification	Species						
	D. caucasica	*D. cneorum*	*D. giraldi*	*D. jasminea*	*D. laureola*	*D. retusa*	*D. tangutica*
No PGR Supplement	0 ±0	0±0	0±0	0±0	0±0	0±0	0±0
1/2 strength salts	0 ±0	6.67±0.24	0±0	15.56±2.4	6.67±1.667	6.67±0.8	6.67±1.6
1/2 sucrose	0 ±0	0±0	0±0	6.67±0.00	0±0	0±0	0±0
0.001 mg l^{-1} IAA	0 ±0	3.33±0	0±0	8.89±3.8	11.11±3.8	0±0	0±0
0.01 mg l^{-1} IAA	13.33±6.6	6.67±3.8	17.78±3.1	26.67±2.4	20±1.1	0±0	0±0
0.1 mg l^{-1} IAA	15.56±3.8	6.67±0	22.22±3.84	40±3.8	33.33±6.6	11.11±3.8	20.00±2.4
0.5 mg l^{-1} IAA	2.22±3.8	17.78±3.8	31.11±1.05	26.67±1.2	13.33±0.8	17.78±3.8	20.00±3.8
0.01 mg l^{-1} IAA+ 1 mg l^{-1} Charcoal	13.33±6.6	22.22±0.6	13.33±1.67	46.67±2.8	20±2.4	26.67±0.8	13.33±1.1
0.001 mg l^{-1} NAA	42.22±3.8	28.89±6.6	40±2.0	37.78±3.8	41.11±4.6	26.67±1.8	35.56±4.3
0.01 mg l^{-1} NAA	37.78±3.8	33.33±3.8	43.33±2.3	48.89±3.8	40.00±0	33.33±3.8	33.33±1.6
0.1 mg l^{-1} NAA	24.44±3.8	15.56±3.8	28.89±0	41±1.5	34.44±3.8	38.25±4.1	43.44±2.4
0.5 mg l^{-1} NAA	31.11±3.8	13.33±6.6	26.67±1.3	26.67±1.2	33.33±2.4	22.22±3.8	26.67±3.8
0.01 mg l^{-1} NAA+ 1 mg l^{-1} Charcoal	46.67±0.6	33.33±0	44.44±3.8	46.67±3.8	46.67±3.8	46.67±2.6	51.11±2.1
0.001 mg l^{-1} IBA	31.11±3.8	8.89±3.8	40± 2.4	37.78±2.7	44.44±3.8	33.33±0.8	26.67±3.8
0.01 mg l^{-1} IBA	35.56±3.4	31.11±3.8	33.33±1.4	48.89±3.8	33.33±2.3	40.00±4.1	26.67±1.6
0.1 mg l^{-1} IBA	17.78±3.8	22.2±1.4	24.44±3.8	46.67±2.1	33.33±6.6	20.00±1.6	17.78±2.4
0.5 mg l^{-1} IBA	20.00 ±0.6	8.89±3.8	13.33±1.5	40.00±1.6	13.33±1.5	40.00±3.8	26.67±2.1
0.01 mg l^{-1} IAA+ 1 mg l^{-1} Charcoal	35.56±3.8	24.44±0	35.56±3.8	44.4±3.6	35.56±3.8	31.11±2.6	22.22±1.6
0.1 mM l^{-1} NAA+ 0.1 mM l^{-1} IAA	42.22±3.8	37.78±3.8	40± 2.1	46.67±3.6	35.56±3.8	33.33±3.1	40.00±3.8
0.1 mM l^{-1} NAA+ 0.1 mM l^{-1} IBA	53.33±6.6	41.44±3.8	40±3.8	76.14± 2.4	42.18±2.6	46.67±2.4	53.33±2.6
0.1 mM l^{-1} IAA+ 0.1 mM l^{-1}IBA	22.22±3.8	28.89±2.4	24.44±3.8	40.23±1.3	28.89±3.8	22.22±3.8	13.33±1.1
1 mM l^{-1} IBA+ 1 mM l^{-1} NAA	42.22±3.8	35.56±3.8	44.44+2.4	78.00±3.8	39.88±3.8	40.00±2.8	51.11±3.8
Average	**23.94±1.1**	**20.24±0.8**	**25.6±1.5**	**37.76±2.6**	**27.56±3.8**	**24.36±2.1**	**23.99±1.6**
F value (21,44) $_{0.05}$ =	**37.3**	**39.7**	**31.8**	**66.2**	**44.3**	**31.1**	**29.6**

Table 3.5. *Daphne* species rooting frequency (%) after 8 weeks of culture on 2 layered media incorporating auxins in one of the basal medium layers and no PGRs in the second layer); mean ± SD.

Treatment	Species name						
Layers (L_1 / L_2)	*D. caucasica*	*D. cneorum*	*D. giraldi*	*D. jasminea*	*D. laureola*	*D. retusa*	*D. tangutica*
(BM) / (BM+ 0.001 NAA)	62.2±3.8	40±6.6	68.8±3.8	69.6±6.6	60±3.8	48.2±5.3	60±3.8
(BM) / (BM+ 0.01 NAA)	66.6±6.6	46.6±0.8	60±6.6	87.3±3.8	57.7±3.8	61.8±3.4	66.6±3.8
(BM) / (BM + 0.1 NAA)	57.7±3.8	42.2±3.8	60±2.6	80±15.3	46.6±1.9	52.4±3.8	60±6.6
(BM) / (BM + 0.001 IBA)	46.6±6.6	26.6±1.4	40±3.8	36.6±1.9	53.3±3.8	40±1.9	33.3±1.9
(BM) / (BM+ 0.01 IBA)	42.2±3.8	40±2.6	51.1±3.8	40±3.8	46.6±1.9	46±3.8	46.6±3.8
(BM) / (BM+ 0.1 IBA)	40±1.9	26.6±6.6	42.2±3.8	20±1.4	44.4±3.8	46±3.8	35.5±6.6
(BM+ 0.01 NAA) / (BM)	44.4±3.8	33.3±1.4	46.6±2.6	35.5±3.8	42.2±3.8	26±1.9	35.5±3.8
(BM+ 0.01 IBA) / (BM)	42.2±2.4	28.8±3.8	42.2±1.9	37.7±3.84	33.3±1.9	20±3.8	27.7±1.9
Average	**47.2±3.8**	**35.5±1.4**	**48.4±2.7**	**52.2±1.9**	**48±2.7**	**42.8±1.9**	**45.7±3.8**

* BM (Basic medium) was different for different species based on their primary response; for *D. cneorum* and *D. jasminea* WPM was used while the remaining species used MS.

Table 3.6. Rooting frequency after 8 weeks of culture on basal medium following treatment with auxin solution dip treatment; mean ± SD

Treatment	Species name						
A. variable time dip	*D. caucasica*	*D. cneorum*	*D. giraldi*	*D. jasminea*	*D. laureola*	*D. retusa*	*D. tangutica*
IBA (1mM) [5 min]	20±6.6	13.33±6.6	8.89±3.8	13.33±1.8	15.56±3.8	17.78±3.8	6.667±2.6
IBA (1mM) [15 min]	22.22±3.8	40.00±1.8	17.78±3.8	40.00±6.6	20.00±1.4	20.00±1.4	20.00±6.6
IBA (1mM) [30 min]	40±6.6	28.89±3.8	26.67±5.1	44.44±3.8	33.33±2.4	33.33±6.6	26.67±3.8
NAA (1mM) [5 min]	20±6.6	22.22±3.8	26.67±1.8	26.67±2.4	40.00±6.6	40.00±6.6	26.67±6.6
NAA (1mM) [15 min]	44.44±3.8	48.89±3.8	53.33±6.6	60.00±15.3	53.33±15.3	60.00±15.3	53.33±11.4
NAA (1mM) [30 min]	44.44±2.6	53.33±6.6	44.44±3.8	46.67±6.6	48.89±3.8	60.00±8.4	46.67±5.1
B. 15 min dip							
IBA (10mM) + NAA (10 mM)	44.44±3.8	26.67±1.4	46.67±6.6	77.78±15.3	40.00±8.4	66.67±5.1	59.31±5.1
IBA (10mM) + NAA (1mM)	62.22±15.3	79.93±2.6	58.14±15.3	89.18±11.4	53.33±6.6	64.44±3.8	57.10±11.4
IBA (10mM) + NAA (0.1mM)	37.78±3.8	42.22±3.8	42.22±3.8	66.67±5.1	40.00±2.4	53.33±6.6	46.67±6.6
IBA(10mM) + NAA(0.01mM)	37.78±3.8	37.78±3.8	26.67±2.6	55.56±3.8	46.67±15.3	53.33±11.4	40.00±2.4
IBA(1mM) + NAA(10 mM)	26.67±2.6	42.22±3.8	26.67±3.8	46.67±2.6	24.44±3.8	33.33±5.1	44.44±3.8
IBA(1mM) + NAA(1mM)	55.56±5.1	60.00±6.6	57.78±3.8	73.33±8.4	46.67±2.6	33.33±2.6	46.67±6.6
IBA(1mM) + NAA(0.1mM)	53.33±6.6	51.11±3.8	53.33±11.4	75.56±3.8	60.00±6.6	46.67±8.4	53.33±8.4
IBA(1mM) + NAA(0.01mM)	35.56±3.8	37.78±3.8	33.33±2.6	53.33±6.6	46.67±5.1	44.44±3.8	40.00±2.6
IBA(0.1mM) + NAA(10mM)	26.67±2.6	48.89±6.6	24.44±3.8	46.67±2.6	20.00±1.4	31.11±3.8	33.33±6.6
IBA(0.1mM) + NAA(1mM)	33.33±6.6	40.00±2.6	28.89±3.8	48.89±3.8	33.33±8.4	33.33±1.4	37.78±3.8
IBA(0.1mM) + NAA(0.1mM)	35.56±3.8	33.33±6.6	46.67±15.3	44.44±3.8	44.44±3.8	26.67±2.6	28.89±3.8
IBA(0.1mM) + NAA(0.01mM)	33.33±6.6	46.67±3.8	31.11±3.8	46.67±6.6	53.33±6.6	22.22±3.8	26.67±5.1
IBA(0.01mM) + NAA(10 mM)	48.89±3.8	46.67±6.6	48.89±5.1	40.00±8.4	42.22±3.8	46.67±15.3	53.33±11.4
IBA(0.01mM) + NAA(1mM)	48.89±3.8	33.33±1.4	46.67±2.4	40.00±3.8	33.33±8.4	40.00±8.4	46.67±6.6
IBA(0.01mM) + NAA(0.1mM)	22.22±3.8	33.33±6.6	31.11±3.8	35.56±3.8	40.00±2.4	20.00±6.6	31.11±3.8
IBA(0.01mM) + NAA(0.01mM)	22.22±3.8	26.67±3.8	6.67±0	40.00±6.6	37.78±3.8	13.33±3.8	22.22±3.8
Average	**37± 2.7**	**40.6±3.8**	**35.7±2.4**	**50.5±5.1**	**39.7±2.7**	**39±3.8**	**37.5±2.4**

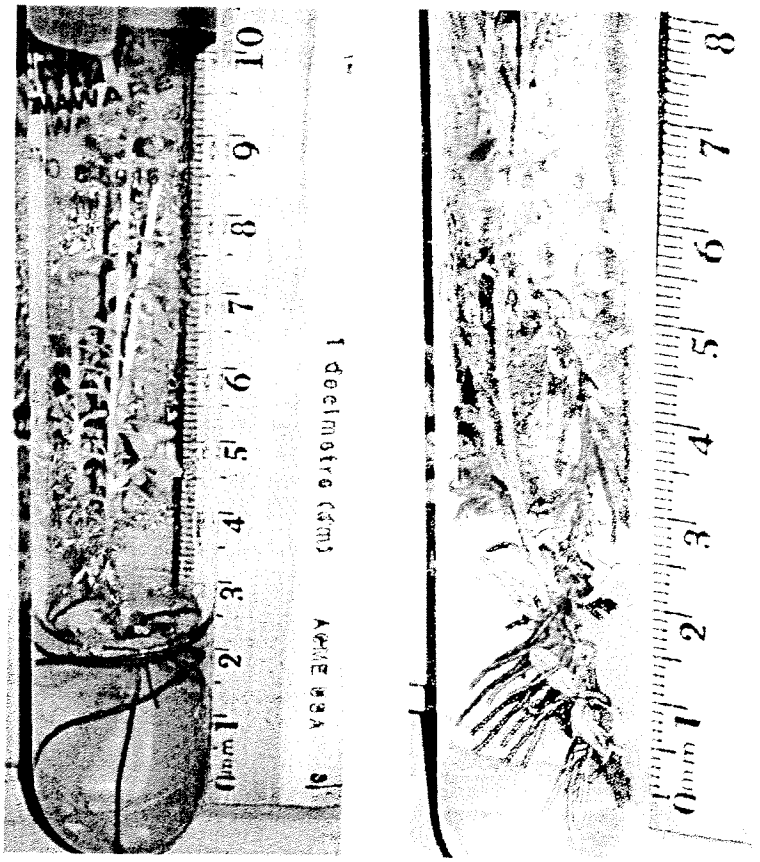
Table 3.6. Rooting frequency after 8 weeks of culture on basal medium following treatment with auxin solution dip treatment; mean ± SD-

continuation from the previous page

F (21,44) = (P< 0.05)	23.29	48.15	101.47	61.08	104.16	225.31	108.86

Figure 3.1. Multiplication stage of *Daphne* species

Figure 3.2. Root formation of *Daphne* microshoots after 8 weeks on WPM media.



CHAPTER FOUR [1]

EVALUATION OF DAPHNE GERMPLASM FOR RESISTANCE TO DAPHNE SUDDEN DEATH SYNDROME (DSDS) CAUSED BY THE SOIL-BORNE PATHOGEN *THIELAVIOPSIS BASICOLA*

4.1 Introduction

The genus *Daphne* L. (Thymelaeaceae Juss.) is comprised of approximately 95 species (Flora of China, 2005) distributed through Africa, Northern and Southern Europe, the Middle East, Asia and regions of Oceania. Of these species, several have been commercialized because of their many desirable horticultural characteristics including attractive foliage, plant habit, flower color, and most of all, pleasant fragrance. Specifically, *D. cneroum* L. (Rose daphne or garland flower) has become one of the most popular perennial flowering shrubs among ornamental plant growers. However, the genus has acquired a poor reputation because of poor long-term performance of this and other *Daphne* introductions. One of the major limitations to daphne's survival in cultivation is Daphne Sudden Death Syndrome (DSDS), a disease caused by the fungal root pathogen *Thielaviopsis basicola* (Berk. & Br.) Ferraris (syn. *Chalara elegans* Nag Raj et Kendrick) (Noshad et al. 2006).

---

1 A version of this chapter has been accepted for publication in Hortscience.

Noshad D, Punja ZK, Riseman A. Evaluation of *Daphne* germplasm for resistance to Daphne Sudden Death Syndrome caused by the soil-borne pathogen *Thielaviopsis basicola*

This disease kills plants quickly, as the name suggests, following the first foliar symptoms. Observations on DSDS-infected plants indicate the following progression of symptoms: (i) brown to black necrotic lesions on the roots, (ii) leaf chlorosis leading to abscission, (iii) whole plant stunting, and (iv) stem collapse and plant death (Noshad et al, 2006).

Thielaviopsis basicola is a widespread root fungal pathogen found in both agricultural and nonagricultural soils (Anderson and Welacky, 1988; Trojak-Goluch and Berbec, 2005; Hood and Shew, 1996). A wide range of economically important plants are infected by *T. basicola* including carrot (*Daucus carota* L.) (Punja etal, 1992), cotton (*Gossypium hirsutum* L.) (Wheeler etal, 1999), and tobacco (*Nicotiana tabacum* L.) (Hood and Shew, 1997b & 1996). Infection by *T. basicola* commonly results in a form of black root rot disease, whole plant stunting, and delayed maturity, all of which reduce yield and crop quality. This pathogen is generally considered a facultative parasite, recently described (Reddy, 1989; Walker etal, 1999; Hood and Shew, 1997a&b) as a hemibiotrophic pathogen. This designation refers to *T. basicola*'s ability to survive for long periods of time outside of a living host by saprophytic consumption of soil organic matter (Hood and Shew, 1997b; Punja etal, 1993; Shew and Meyer, 1992). The fungus produces two spore types, phialospores (endoconidia) and chlamydospores (aleuriospores). The chlamydospore is characterized by a thick pigmented wall and divisions into compartments or segments, each of which is able to germinate. The phialospores are hyaline, not septate, and have a rectangular shape. The taxonomic classification of *T. basicola* is as follows (Nag Raj and Kendrick, 1975):

Kingdom: Fungi

Division: Eumycota

Subdivision: Deuteromycotina (Fungi Imperfecti)

Class: Moniliales

Genus: *Chalara*

Species: *Thielaviopsis basicola* (syn. *Chalara elegans*)

The literature indicates that variability in virulence, due to both external and internal factors, exists within *T. basicola*. For example, *T. basicola* is reported to be most aggressive during the growing season when soil temperatures are ≤ 24°C (Walker etal, 1999) and soil water content is high (Rothrock, 1992; Walker et al, 1999). Bottacin et al. (1994) described variation in virulence among several isolates of *T. basicola* and related this to their genetic composition. Specifically, they described the existence of dsRNAs in some, but not all, isolates of *T. basicola* (Bottacin et al., 1994) and related this to changes in the level of virulence (Park etal, 2005; Punja etal, 1995; Geldenhius etal, 2004). In support, it has been shown that strains without dsRNA were less pathogenic than strains with one dsRNA strand and that these were more pathogenic than strains with more than one dsRNA strand (Punja, 1995). As a result, the authors have concluded that the presence of this particular dsRNA in *T. basicola* increases the pathogenicity of this fungus (Punja, 1995; Park etal, 2005). Most recently, Park et al (2005) examined infection of *T. basicola* by two totivirus-like dsRNAs. A full-length cDNA clone was developed from the 5.3 kb dsRNA element present. Sequence analysis revealed that it contained three large putative open reading frames (ORFs). These results indicate that the presence of putative virus-like particles in the cytoplasm, which were

similar in both shape and size to viruses in the Totiviridae, increase the virulence in certain isolates of *T. basicola* (Punja, 1995; Park etal, 2005; Wattimena, 2001).

Observations on the interactions of *T. basicola* with other organisms show that *T. basicola* can be sensitive to antagonism (Howell, 2003, Hood and Shew, 1997b). For example, *Pseudomonas* spp. populations in the rhizosphere of tobacco roots produced biocontrol compounds (e.g., hydrogen cyanide (HCN)) suppressive to *T. basicola* (Troxlert and berling, 1997). The antagonistic activity of *Trichoderma* spp. against many pathogens, including *T. basicola*, have also been reported (Howell etal, 2003). However, biocontrol using *Trichoderma* against *T. basicola* has not yet been approved for commercial applications (Howell etal, 2003). In another study, a synergism between the root-knot nematode, *Meloidogyne incognita* (Kofoid & White), and *T. basicola,* was identified. When occurring together, these pests greatly facilitate the development of black root rot in cotton; a seedling disease that is characterized by severe rotting of the cortex of young cotton hypocotyls and roots (Walker etal, 1999). Evaluating genetic variation in *T. basicola* using ISSR-PCR, seven of fourteen primer pairs resulted in the amplification of single polymorphic fragment indicating quantifiable variation is present among populations (Geldenhuis etal., 2004). Continued use of these primers will enable further molecular characterization of this important pathogen resulting in an enhanced understanding of its population structure (Geldenhuis et al, 2004).

Little is known about the factors (e.g., cultural conditions, host plant genetics) that affect DSDS development beyond anecdotal observations and practices. For example, during

nursery production of daphne cultivars, many producers apply prophylactic fungicide treatments to help ensure crop health. While this is a relatively common practice, no published literature was found that directly addresses the efficacy of this practice to control DSDS. In addition, even if effective at controlling DSDS, reliance on fungicides is unsustainable and undesirable. One alternative to this practice is the development of disease resistant daphne cultivars. This strategy is typically more desirable because it can be highly effective in reducing disease, is environmentally benign, and usually entails little or no additional expense to producers (Crute, 1996; Dahlberga, 2001; Diaz-Pérez 1995; Reeleder, 1999). However, it typically requires a long time horizon to achieve. To date, native host plant resistance to *T. basicola* has been identified during germplasm screens as part of various crop improvement or breeding programs. For example, *Nicotiana glauca* Graham was identified as resistant to *T. basicola* and subsequently incorporated into a tobacco (*N. tabacum*) breeding program (Trojak-Goluch and Berbec, 2005). In another germplasm screen, *Gossypium arboreum* L. PI 1415 was found to be resistant to *T. basicola* and subsequently incorporated into a diploid cotton breeding program (Berbec, 1976; Wheeler, 1999; Shankara, 1999). Based on these reports, evaluating *Daphne* germplasm for resistance to DSDS, via controlled screens, is a reasonable approach to identify host plant resistance to this pathogen. Therefore, the objectives of this study were: 1) to develop an efficient method for evaluating resistance of *Daphne* taxa to *T. basicola*; 2) to compare *in vivo* and *in vitro* methods for their efficiency in identifying resistance of selected *Daphne* taxa; 3) to develop a useful disease progression index (DPI) for use in taxa evaluations; and 4) to rank *Daphne* germplasm for resistance to *T. basicola*.

4.2 Materials and methods

4.2.1 Plant material

Thirty-two species and cultivars of *Daphne* were collected and maintained at the UBC Botanical Garden and Center for Plant Research, Vancouver, BC, Canada. All 32 taxa were included in an in vivo challenge while a subset of seven species was used in an in vitro challenge (Table 4.1). Container-grown stock plants were used to supply tissue for both in vitro tissue culture establishment and traditional vegetative propagation. Rooted plants were produced in July and August from terminal cuttings (50-100 mm in length) with the flower buds and lower leaves removed. Cuttings were made with a single shallow cut and soaked in an anti-fungal solution (Physan 20, Maril Products Inc., Tustin, CA) for 60 seconds. The cuttings were allowed to dry momentarily before being dipped in 0.4% IBA powder (Stim Root #2, Plant Products. Co. Ltd., Brampton, Ontario) and then placed in 6 cm pots filled with a course rooting medium (10 parts propagation grade perlite, 8 parts peat, 6 parts granite grit #2, 1 part pumice (double screened to remove fine particles), dolomite lime 65AG at 900 g m^{-3}, and Micromax (trace elements) at 400 g m^{-3}). The flats were placed under intermittent mist with bottom heat set at 22°C. Rooted cuttings were transferred to a polyhouse in October where they were allowed to go dormant but kept frost free. They were repotted in May into 12 cm pots filled with a well-drained medium (8 parts peat, 8 parts Turface MVP, 6 parts granite grit #2, 4 parts screened and pasteurized soil, 1 part pumice, dolomite lime 65AG at 670 g m^{-3}, Micromax micronutrients at 540 g m^{-3}, Osmocote 18-6-12 at 2150 g m^{-3}, and Psi Matric wetting agent). All stock plants were grown under shade cloth during the summer months and moved to a heated polyhouse during the winter months to prevent frost damage. Fertilization regiment included yearly top-dressing with Osmocote 18-6-12 at 5 g l

gal pot. Fungicides were not used during stock production because we had very little disease pressure and we did not want to risk cross-contamination affecting the in vivo assay.

4.2.2 Pathogen culture and suspension preparation

A single aggressive pathogenic isolate of *T. basicola* was cultured from diseased daphne plants and used throughout this study (Noshad et al., 2006). A suspension of endoconidia was prepared by gently washing the surface of 3-week-old colonies with deionized water and vortexing the wash solution for 30s. The resulting suspension was twice filtered through four layers of cheesecloth to remove agar, hyphae, and chlamydospores. The spore suspension was calibrated with a haemocytometer and adjusted with deionized water to obtain a final concentration of endoconidia of $1x10^6$ ml^{-1} prior to inoculation. A preliminary experiment was conducted to determine the optimum concentration of pathogen spores for effective inoculation. The results of this experiment indicated that a concentration of $1x10^6$ ml^{-1} induces infection within a reasonable time (Appendix 1).

4.2.3 *In vivo* challenge

The conidial suspension (5 ml) was topically applied to healthy roots of 2-year old nursery-grown plants. Production containers were modified to contain a clear panel behind a lightproof 'door' to allow for direct observation of the infection process without further disturbance to the root system (Fig 4.1). All procedures were the same for control plants except for the application of distilled water instead of the spore suspension. To allow for uniform conditions following inoculation, plants were transferred to a greenhouse and grown under natural light at 25 $\pm$1 °C and a relative humidity between 70 and 80%.

4.2.4 *In vitro* challenge

Seven of the 32 taxa were selected for inclusion in an *in vitro* challenge (Fig 4.2). Clean cultures of these taxa were established and axillary shoot proliferation obtained from nodal explants cultured on either MS (Murashig and Skoog, 1962) or WPM (Woody Plant Medium; McCown and Lloyd, 1983) supplemented with plant growth regulators (i.e., 2 mg l^{-1} BA + 0.01 mg l^{-1} NAA), recommended minerals and 5.6 g l^{-1} agar based on these two major protocols (Murashig and Skoog, 1962; McCown and Lloyd, 1983). All the *in vitro* plant cultures were maintained at 24°C with a 16/8 h photoperiod supplied by cool white fluorescent lamps delivering 350 μW cm^{-2}. Axillary shoots were rooted by subculturing on WPM/MS medium with vitamins (nicotinic acid 0.5 g l^{-1}, pyridoxine-HCl 0.5 g l^{-1}, thiamine-HCl 1 g l^{-1}, and glycine 2 g l^{-1}), 20 g l^{-1} sucrose, and 5.6 g l^{-1} agar following dipping in plant growth regulators filter sterilized solution i.e. IBA (10 mM) + NAA (1 mM). Following six to eight weeks, shoots were rooted and ready for inoculation. Using a 1 ml syringe, 1 ml of the conidial suspension was injected next to a root segment while still embedded in the culture medium (Fig 4.1). All control plants were treated equally to the test plants except for injection of distilled water instead of the conidial suspension (Svabova, 2005; Jarausch, 1999).

4.2.5 Disease assessment

For both methods, observations on disease progression following inoculation were made weekly over eight weeks. The following disease progression rating (DPR) was developed: 0 = healthy plant, no symptoms; 1 = less than five lesions on lateral roots, no lesions on tap

root, no foliar symptoms; 2 = greater than five lesions on lateral roots, less than five lesions on tap root, no foliar symptoms; 3 = most lateral roots with lesions and some necrosis, greater than five lesions on tap root, five to ten chlorotic leaves; 4 = most lateral roots necrotic, greater than five lesions on tap root, most leaves chlorotic with some leaf abscission; 5 = plant is dead. A plant disease index (PDI) was developed based on both disease incidence (DI) and disease severity (DS) as follows:

$$PDI = DS/L \times DI/N \times 100$$

where DS= disease severity (i.e., average DPR value among diseased plants); L= the number of DPR disease categories; DI= disease incidence (i.e., number of diseased plants); and N= total number of plants. This approach allows for greater resolution of absolute taxon susceptibility by combining both relative disease progression within individuals with the absolute occurrence of the disease within a taxon. Therefore, based on this assessment, a completely susceptible taxon would score 100 while a completely resistant taxon would score 0.

4.2.6 Data analyses

Both methods used a completely randomized design with three replications. Each replicate consisted of 24 plants per taxon with two plants serving as controls. Descriptive statistics, analysis of variance, correlation, and Tukey's honest significant differences (HSD) test were generated using SPSS 11.5 software (SPSS Inc. Statistical Package for the Social Sciences, Chicago, US).

4.3 Results

4.3.1 *In vivo* screen

Significant differences ($P<0.05$) among taxa were observed for PDI values of *T. basicola* infection. Eight weeks following inoculation, PDI values ranged from 64.6 for *D. cneorum* to 0 for *D. tangutica* and *D. retusa* (Table 4.1). For all other taxa, PDI values ranged between these extremes and with varying levels of chlorosis, leaf abscission, and stunting observed. Typical disease progression in susceptible taxa can be generalized as follows: 1-3 weeks post-inoculation- brown to black necrotic lesions on the roots; 4-6 weeks post inoculation- leaf chlorosis leading to abscission; and 6-8 weeks post-inoculation- whole plant stunting, stem collapse and plant death. However, for highly resistant taxa, no visible infection or discoloration developed at any time.

4.3.2 *In vitro* screen

Similar to the *in vivo* challenge, significant differences between the susceptible and resistant taxa evaluated were identified for *T. basicola* development. As in the *in vivo* screen, *D. cneorum* was the most susceptible to *T. basicola* infection (PDI = 72.2) while *D. tangutica* and *D. retusa* were the most resistant (PDI = 3.3 and 3.6, respectively) (Fig 4.2). For the five remaining taxa, PDI ranged between these extremes and with varying levels of chlorosis, leaf abscission, and stunting observed (Fig 4.2).

4.3.3 Assay comparison

For the seven taxa included in both assays, strong similarities were present for overall taxa performance while a significant difference in the timing of disease progression was present. A high correlation coefficient (R=0.87) was calculated between these systems for PDI values, indicating both systems were comparable in evaluating disease susceptibility among these seven taxa. In addition, the rank order of taxa based on DPI values for the two methods were identical, further supporting these methods as equal in evaluating disease susceptibility. However, the *in vitro* system produced results in significantly less time. On average, three fewer weeks were required to reach the same level of disease progression as compared to the *in vivo* system.

4.4 Discussion

The identification and incorporation of host-plant resistance into susceptible plants is an often sought-after goal for many breeding programs. It has been successfully achieved using both conventional breeding as well as biotechnological methods. However, despite significant differences between these two approaches, both rely on a robust germplasm screen to differentiate germplasm performance following pathogen exposure. A screen incorporated into a traditional breeding program often involves the evaluation of progeny derived from hybridizations between resistant and susceptible parents or the evaluation of related taxa if resistance was not present in the most advanced gene pool (Punja, 2001; Agrios 2005; Iglesias, 2000; Daryonol 2005). This approach has been successfully used to transfer *T. basicola* resistance between related species. In independent breeding programs, *T. basicola* resistant tobacco genotypes were developed based on germplasm screens of related

Nicotiana species (Bai et al. 1996; Palakarcheva 1995; Trojak-Goluch and Berbec 2005). Specifically, the use of a robust germplasm screen allowed researchers to identify *T. basicola* resistance in *Nicotiana debneyi* (Bai et al. 1996; Wilkinson, 1991) and to further conclude it was conditioned by a single dominant gene. Once transferred to a susceptible genetic background, this gene conferred the same degree of resistance as found in the original *N. debneyi* accession (Legg, 1981; Palakarcheva 1995, Bai et al. 1996; Keller, 1999). In a *Gossypium* germplasm screen designed to identify *T. basicola* resistance, significant variation was observed among taxa, with the strongest resistance identified in *Gossypium arboreum*. This resistance was then successfully transferred to commercial cotton cultivars (Walker 1999; Weeler et al, 1999; Rothrock, 1992). Development of a robust *T. basicola* germplasm screen is valuable in not only identifying taxon-specific variation for pathogen resistance but also for further evaluation of the plant's genetic structure. Among the 32 *Daphne* taxa evaluated, significant differences were present for resistance to *T. basicola* under both *in vivo* and *in vitro* challenges. Of the 32 taxa, *D. tangutica* and *D. retusa* displayed the greatest resistance and remained symptom-free during the *in vivo* challenges, while displaying only mild symptoms in the *in vitro* challenges. At the other extreme, *D. cneroum* was clearly the most susceptible taxon in both screens and became fully diseased followed by plant death in the shortest amount of time. The observed range in disease resistance among *Daphne* taxa indicates my challenge was effective and identifies *D. tangutica* and *D. retusa* as potential sources for resistance.

The inoculation and screening methods were proven robust in terms of the isolate's pathogenicity, disease characterization, consistency over time, and in its ability to

differentiate taxa. Based on the one isolate used, overall pathenogenicity was adequate in allowing assessment of disease incidence and severity (i.e., root and foliar symptoms) on all taxa. Also, the concentration of conidia used was sufficient to cause disease but not to overwhelm the defense mechanism(s) and prevent taxa differences from being displayed. Finally, both screening methods produced comparable and consistent results over an 11-month period despite the *in vivo* challenge being conducted in an outdoor polyhouse exposed to seasonal fluctuations. These observations validate our screen methodology and support its continued use in identifying genetic variation among *Daphne* species for resistance to DSDS.

The results of the *in vitro* and *in vivo* experiments indicate a strong correlation between these two assay methods. However, there were differences in disease progression rates between them. Typically, the *in vitro* challenge produced a comparable level of disease as the *in vivo* challenge but in two to three weeks less time. Differences in disease development rates between plants produced from tissue culture and traditional propagation have been reported and may be based on anatomical (e.g., root structure), biological (e.g., adaptation mechanisms with other organisms) or physiological (e.g., difference in biochemical compounds) differences (Diaz-Pérez, 1995). In addition, there was a small temperature difference between my two assay environments with the *in vitro* assay conducted at a higher temperature than the previously reported optimum temperature for *T. basicola* (Walker et al., 1999). Based on 100% infection of *D. cneorum*, the most susceptible taxon, and rebust growth of the pathogen, I conclude this higher temperature is still within the effective range needed for infection.

One limitation of this research was the use of a single isolate of *T. basicola*. The use of a single isolate limits the breath of conclusions that can be made. Various isolates of *T. basicola*, from different regions, may have genomic differences that could affect my assay results. Resistance to one isolate cannot be assumed to extend to all races of this pathogen. It is possible that some of the *Daphne* taxa could react differently to other races. These other races could have different virulent genes (based on gene-for-gene theory) that interact with another type of resistance gene in *Daphne*. However, despite this limitation, for the one aggressive isolate used, significant taxa differences were observed for resistance.

In future studies, additional races of *T. basicola* should be collected and used in the germplasm screen to increase our knowledge about how these two organisms interact. This research may reveal important information on the pathogen's infection process and the various factors that influence both successful infection and successful defense.

4.5 References

Adams PB and GC Papavizas (1969) Survival of root-infecting fungi in soil. Phytopathology. 59, 135-138.

Agrios GN (2005) Plant Pathology. 5th ed. Academic Press, Elsevier Science and Technology books, NY.

Anderson TR and TW Welacky (1988) Populations of *Thielaviopsis basicola* in burley tobacco field soils and the relationship between soil inoculum concentration and severity of disease on tobacco and soybean seedlings. Can. J. of Plant Pathol. 10, 246-251.

Arseniuk E and PC Czembor (1999) Host–parasite interactions: *Septoria* and *Stagonospora* Diseases of Cereals: A Compilation of Global Research. Mexico, DF:CIMMYT, 63-70.

Bai DP, Reeleder R and JE Brandle (1996) Production and characterization of tobacco addition lines carrying *N. debneyi* chromosomes with a gene for resistance to black root rot. Crop Sci. 36, 852-857.

Berbec J and A Berbec (1976) Growth and development of *Nicotiana tabacum* L. from reconstituted on the cytoplasm of *Nicotiana glauca* Grah. Gen. Polonica 17, 309-318.

Bottacin AM, Lévesque CA, and Punja ZK (1994) Characterization of dsRNA in *Chalara elegans* and effects on growth and virulence. Phytopathology. 84, 303-312.

Crute IR and Pink DA (1996) Genetics and utilization of pathogen resistance in plants. The Plant Cell 8, 1747-1755.

Dahlberga JA and Bandyopadhyay R (2001) Evaluation of sorghum germplasm used in US breeding programmes for sources of sugary disease resistance. Plant Pathol. 50, 681-689.

Daryonol BS and Natsuaki KT (2005) Screening for resistance to Kyuri green mottle mosaic virus in various melons. Plant Breeding 124, 487-490.

Diaz-Pérez J (1995) Acclimatization and subsequent gas exchange, water relations, survival and growth of microcultured apple plantlets after transplanting them in soil. Physiologia Plantarum 95, 225-229.

Flora of China Vol. 13 (*Clusiaceae through Araliaceae*). (2005) Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Gayed SK (1972) Host range and persistence of *Thielaviopsis basicola* in tobacco soil. Can. J. Plant Sci. 52, 869-873.

Geldenhius M, Roux J, Wingfield M and Wingfield B (2004) Development of polymorphic markers for the root pathogen Thielaviopsis basicola using ISSR-PCR.
Mol. Eco. Notes. 4, 547–550.

Hood ME and Shew HD (1996) Pathogenesis of *Thielaviopsis basicola* on a susceptible and resistant cultivar of burley tobacco. Phytopathology. 86, 38-44

Hood ME and Shew HD (1997a) Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs. Phytopathology. 87, 228-235.

Hood ME and Shew HD (1997b) Reassessment of the role of saprophyti activity in the ecology of *Thielaviopsis basicola*. Phytopathology. 87, 1214-1219.

Howell C (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Disease. 87, 4-10.

Iglesias A and Pico B (2000) Pathogenicity of fungi associated with melon vine decline and selection strategies for breeding resistant cultivars. Ann. of Appl. Biol. 137,141-151.

Jarausch W and Lansac M (1999) Phytoplasma transmission by *in vitro* graft inoculation as a basis for a preliminary screening method for resistance in fruit trees. Plant Pathol. 48, 283-287.

Jones BL (1991) Penetration and development of *Chalara elegans* in peanut (*Arachis hypogaea*). Phytophylactica 23, 81-84.

Keller H, Pamboukdjian N, Ponchet A, and Poupet A (1999) Induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. The Plant Cell 11, 223-235.

King CJ and Presley J (1942) A root rot of cotton caused by *Thielaviopsis basicola*. Phytopathology. 32, 752-761.

Legg PD and Litton C (1981) Effect of *Nicotiana debneyi* black root rot resistance factor on agronomic and chemical traits in burley tobacco. Theoretical and Appl. Genet. 60, 365-368.

Mims CW, Copes WE and Richardson EA (2000) Ultrastructure of the penetration and infection of pansy roots by *Thielaviopsis basicola*. Phytopathology. 90, 843-850.

Nag Raj TR and Kendrick B (1975) A monograph of *Chalara* and allied genera. Wilfred Laurier University Press, Waterloo, Ontario.

Noshad D, Punja ZK and Riseman A (2006) First report of *Thielaviopsis basicola* on *Daphne cneorum*. Can. J. Plant Pathol. 28, 310-312.

Palakarcheva M (1995) Transfer of disease resistance genes by interspecific hybridization of wild growing *Nicotiana* species in *Nicotiana tabacum*. J. Genet. and Breeding 94, 99-105.

Papavizas GC (1968) Survival of root-infecting fungi in soil. Phytopathology. 58, 421-428.

Park Y, James D and Punja ZK (2005) Co-infection by two distinct totivirus-like double-stranded RNA elements in *Chalara elegans* (*Thielaviopsis basicola*). Virus Research. 109, 71–85.

Punja ZK (2001) Genetic engineering of plants to enhance resistance to fungal pathogens, a review of progress and future prospects. Can. J. Plant Pathol. 23, 216-235.

Punja ZK (1995) Influence of double-stranded RNAs on growth, sporulation, pathogenicity, and sumival of *Chalara elegans*. Can. J. Bot. 73, 1001-1009.

Punja ZK, Chittaranjan S and Gaye MM (1992) Development of black root rot caused by *Chalara elegans* on fresh market carrots. Can. J. of Plant Pathol. 14, 299–309.

Punja ZK and Sun LJ (1999) Morphological and molecular characterization of *Chalara elegans* (*Thielaviopsis basicola*), cause of black root rot on diverse plant species. Can. J. Bot. 77, 1801-1812.

Reddy MS and Patrick ZA (1989) Effects of host, nonhost, and fallow soil on populations of *Thielaviopsis basicola* and severity of black root rot. Can. J. Plant Pathol. 11, 68-74.

Reeleder R (1999) Septoria leaf spot of *Stevia rebaudiana* in Canada and methods for screening for resistance. J. Phytopathology. 147, 605-613.

Rothrock CS (1992) Influence of soil temperature, water, and texture on *Thielaviopsis basicola* and black root rot on cotton. Phytopathology. 82, 1202-1206.

Schipper B (1970) Survival of endoconidia of *Thielaviopsis basicola* in soil. Netherlands J. Plant Pathol. 76, 206-211.

Shankara M and Cowling WA (1999) Screening for resistance to *Diaporthe toxica* in lupins by estimation of phomopsins and glucoseamine in individual plants. Plant Pathol. 48, 320-324.

Shew HD and Meyer JR (1992) ThielaviopsisIn: L. L. Singleton, J.D. Mihail, and C. M. Rush, eds. Methods for Research on Soilborne Phytopathogenic Fungi. Amer. Phytopathology, P. 171-174. Soc., St. Paul, MN.

Stover RH (1950a) The black root rot disease of tobacco. I. Studies on the causal organism *Thielaviopsis basicola*. Can. J. of Bot. 28, 445–470.

Stover RH (1950b) Effect of nutrition on growth and chlamydospore formation in brown and gray cultures of *Thielaviopsis basicola*. Can. J. of Bot. 34, 459-472.

Svabova L (2005) In vitro selection for improved plant resistance to toxin-producing pathogens. Phytopathology. 153, 52-64.

Tabachnik M, DeVay JE, Garber RH and Wakeman RJ (1979) Influence of soil inoculum concentrations on host range and disease reactions caused by isolates of *Thielaviopsis basicola* and comparison of soil assay methods. Phytopathology. 69, 974-977.

Trojak-Goluch A and Berbec A (2005) Meiosis and fertility in interspecific hybrids of *Nicotiana tabacum* L., *N. glauca* Grah. and their derivatives. Plant Breeding 126, 201-206.

Troxler J and berling C (1997) Interactions between the biocontrol agent *Pseudomonas fluorescens* CHA0 and *Thielaviopsis basicola* in tobacco roots observed by immunofluorescence microscopy. Plant Pathology. 46, 62-71.

Walker NR, Kirkpatrick TL and Rothrock CS (1999) Effect of temperature on and histopathology of the interaction between *Meloidogyne incognita* and *Thielaviopsis basicola* on cotton. Phytopathology. 89:613-617.

Wattimena S (2001) Studies of factors affecting virulence of *Chalara Elegans* on Bean (Phaseolus Vulgaris L.). MSc. Thesis. Simon Fraser University.

Wheeler MH, Stipanovic RD (1979) Melanin biosynthesis in *Thielaviopsis basicola*. Expt. Mycol. 3, 340-350.

Wheeler TA, Gannaway JR and Keating K (1999) Identification of resistance to *Thielaviopsis basicola* in diploid cotton. Plant Disease. 83, 831-833.

Wick RL and Moore LD (1983) Histopathology of root disease incited by *Thielaviopsis basicola* in *Ilex crenata*. Phytopathology. 73, 561-564.

Wilkinson CA and Rufty RC (1991) Inheritance of partial resistance to black root rot in burley tobacco. Plant Disease. 75, 889-892.

Yarwood CE (1981) Occurrence of *Chalara elegans*. Mycol. 73, 524-530.

Table 4.1. *Daphne* taxa used in the *Thielaviopsis basicola* resistance bioassay with region of nativity or origin and mean plant disease index (PDI) values eight weeks following inoculation.

Taxa	Nativity/Origin	Mean PDI (SD) [z]
D. alpina L.	Italy	12.5 (0.92) [ghi]
D. arbuscula Celak.	Czech Republic	43.3 (1.74) [abcdef]
D. bholua Buch-Ham. ex	Nepal	12.5 (0.82) [ghi]
D. Xburkwoodii 'Carol Mackie'	Horticultural Origin	29.2 (1.32) [cdefgh]
D. caucasica Pall.	Russia	9.2 (1.02) [hi]
D. circassica L.	Russia	46.6 (1.66) [abcde]
D. cneorum L.	Czeck republic	64.2 (1.89) [a]
D. collina Smith	Turkey	51.7 (1.31) [abcd]
D. Xeschmannii	Horticultural Origin	23.3 (1.17) [defghi]
D. genkwa Siebold & Zucc.	China	43.3 (1.31) [abcdef]
D. genkwa (Hackenberry group)	Horticultural Origin	15.0 (1.22) [fghi]
D. ginidium L	Spain	27.5 (1.22) [defgh]
D. giraldii Nitsche	W China	15.8 (1.18) [fghi]
D. jasminea Sibth.& Sm.	Greece	13.3 (1.09) [fghi]
D. kosaninii Stoj.	Bulgaria	20.8 (1.3) [efghi]
D. laureola L.	N Africa	23.3 (1.34) [defghi]
D. 'Lawrence Crocker'	Horticultural Origin	40.8 (1.80) [abcdefg]
D. longilobata Turril.	China-Yunnan	30.8 (1.06) [cdefgh]
D. Xmantensiana	Horticultural Origin	57.5 (1.87) [abc]

D. mezereum L.	Russia	10.0 (1.02) [hi]
D. mezereum (alba)	Horticultural Origin	15.0 (1.32) [ghi]
D. Xnapolitana	Horticultural Origin	39.2 (1.46) [abcdefg]
D. odora Thunb.	China	31.7 (1.21) [cdefgh]
D. oleoides Schreber	Turkey	17.5 (1.19) [fghi]
D. pontica L.	Russia	60.8 (1.54) [ab]
D. retusa Hemsl.	China	0.0 (0) [J]
D. Xrollsdorfii 'Arnold Cihlarz'	Horticultural Origin	25.8 (1.54) [defghi]
D. rossettii Gab.	Horticultural Origin	15.8 (1.06) [fghi]
D. Xthauma	Horticultural Origin	34.2 (1.40) [bcdefgh]
D. tangutica Maxim.	China	0.0 (0) [J]
D. transcaucasica Pobed.	Turkey	19.2 (1.30) [efghi]
D. 'Whilhelm Shacht'	Horticultural Origin	19.2 (1.18) [defghi]

[z] mean values followed by a common letter are not different ($P \leq 0.05$) by Tukey's Honest Significant Differences test.

Figure 4.1. Bioassay containers used for *Daphne* taxa challenged with *Thielaviopsis basicola*; A) in vivo assay pots modified with viewing panel for direct observation of diseased roots; B) in vitro assay with conidial suspension injected next to a root segment while still embedded in the culture medium.

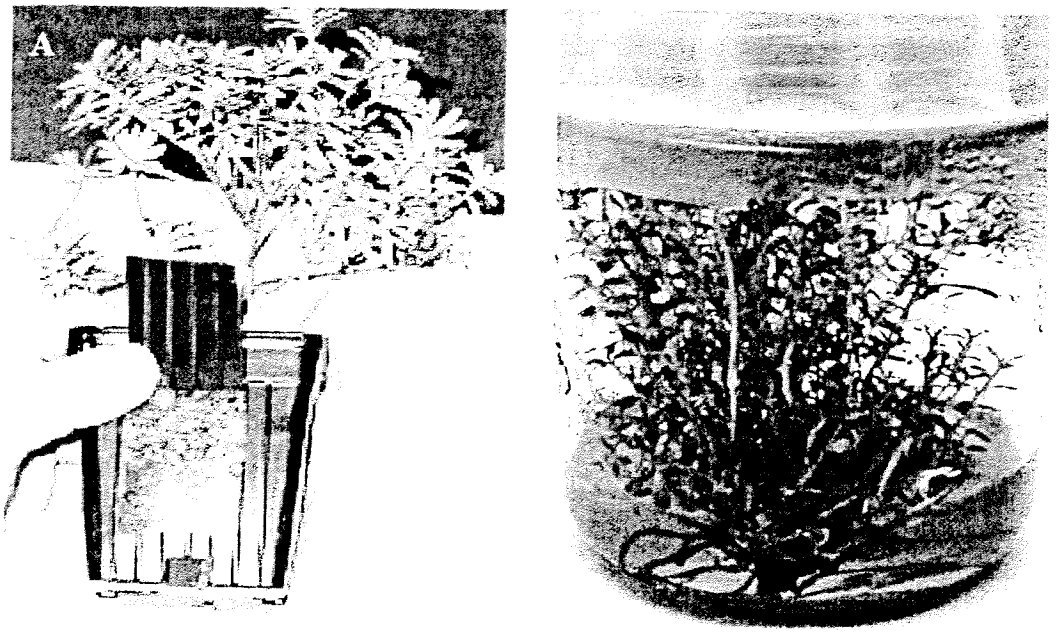
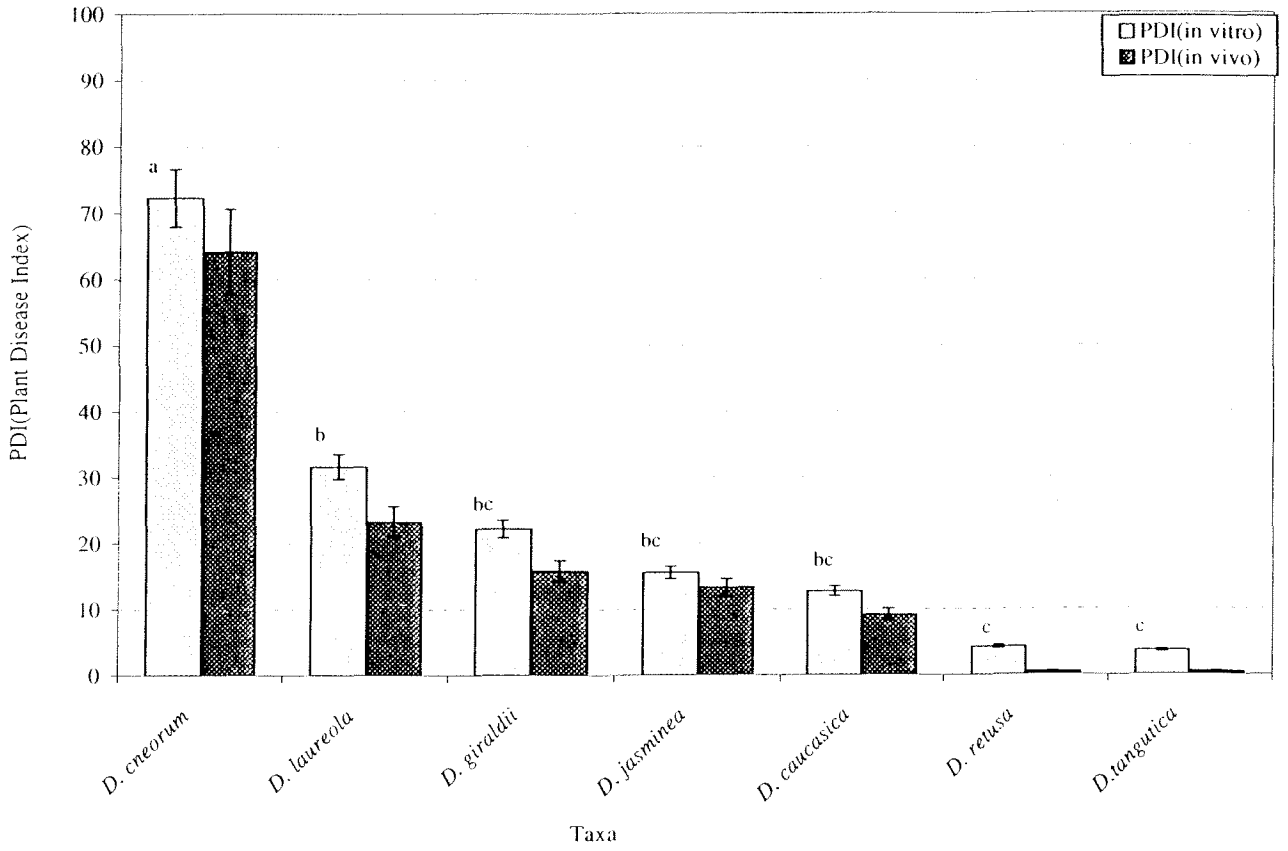


Figure 4.2. Comparison of the *in vitro* with *in vivo* assays using seven *Daphne* taxa and *Thielaviopsis basicola*. Plant disease index (PDI) values are presented at five weeks following inoculation; Bars marked with the same letter do not differ significantly; error bars = SD.



CHAPTER FIVE [1]

COMPARATIVE EVOLUTIONARY ANALYSIS OF rDNA *ITS* SEQUENCES OF SELECTED *DAPHNE* SPECIES WITH REFERENCE TO RESISTANCE TO *THIELAVIOPSIS BASICOLA*

5.1 Introduction

Plants are constantly being subjected to external stressors that require them to respond in an appropriate manner. Among these stressors, the plant's response to pathogen attack has been scrutinized intensely. A thorough understanding of how plants evolved an appropriate response to these stressors (i.e., pathogen resistance) is beneficial not only for our general understanding of how these processes work, but also for the practical application to crop improvement programs (Todd etal, 2000). Following the rediscovery of Mendel's work, plant breeders soon recognized that resistance to disease was often inherited as a single dominant or co-dominant gene (Keen, 1990).

Considerable knowledge has since accumulated on the genetic and biochemical and basis of disease resistance (Hammond-Kosack and Jones, 1996). Most recently, the tools of molecular biology coupled with phylogenetic analysis are now helping researchers to study disease resistance from an entirely new perspective, one of plant evolution.

Molecular sequence data have revolutionized phylogenetic analysis and our understanding of how various organisms are related. Since the late 1980s, molecular-based phylogenetic hypotheses have been proposed for nearly all groups of organisms. With reference to disease resistance, identifying a plant group's phylogenetic relationships can potentially provide insight into how often and under what conditions resistance may have evolved. Karban and Baldwin (1997) speculated that if plants native to environments with a specific endemic pathogen had more resistance than those without the pathogen, phylogenetic analyses could potentially provide insight into the evolution of disease resistance. Unfortunately, no publications relating phylogenetic placement and disease resistance have been identified.

One of the most popular sequences for phylogenetic inference at the generic and infrageneric levels in plants is the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron. Based on a survey of 244 molecular phylogeny papers (Alvarez and Wendel, 2003), two-thirds (66%) included ITS sequence data for comparisons at the genera level or below, and one third (34%) of all published phylogenetic hypotheses have been based exclusively on ITS sequences. The ITS region has become one of the most popular sequences to base phylogenetic inference because of its presumed advantageous properties and availability of comparison sequences (Baldwin et al., 1995). Because the 18S–26S rDNA regions reside in the nuclear genome, ITS sequences are biparentally inherited, and are thus distinguished from the cpDNA loci that are often used for phylogeny construction. Using sequences based on biparental inheritance has been shown critical in revealing past cases of reticulation, hybrid speciation, and parentage of polyploids (Baldwin et al., 1995; Kim and Jansen, 1994; Wendel et al., 1995). With the development of a universal primer set

for amplifying ITS sequences from most plant and fungal phyla (White et al, 1990), ITS sequence data are now more readily obtained than most nuclear-sequenced markers. In addition to these advantages, nuclear ribosomal genes are constituents of individual 18S–5.8S–26S repeats of about 10 kb that are reiterated at one or more chromosomal loci per haploid complement (Baldwin et al., 1995). Because there are hundreds to thousands of nuclear rDNA repeats in plant genomes, they are more easily isolated than most low-copy nuclear loci. In angiosperms, ITS sequences vary in length from approximately 500–700 bp (Baldwin et al., 1995). Based on the ITS advantages of biparental inheritance, ease of sequence generation, and variability in sequence, I have chosen this marker to construct a phylogeny of selected *Daphne* taxa with special reference to resistance against Daphne Sudden Death Syndrome (DSDS) (Noshad et al., 2006*a*).

The genus *Daphne* L. (Thymelaeaceae Juss.) is comprised of approximately 95 species (Flora of China, 2005); although the number of species varies in different sources from 54-95. The genus is distributed through Africa, Northern and Southern Europe, the Middle East, Asia and regions of Oceania. There is no clearly accepted systematic treatment for this group. Over time, authors have defined several number of species and sub genera for this genus highlighting the systematic challenge this group presents (Mathew, 2000; Halda, 2001; Van der Bank, 2002). I used representative species from horticultural groupings, based on morphological traits (Mathew etal, 2000; Halda, 2001).

Several *Daphne* species with horticultural merit (i.e., attractive foliage, plant habit, flower color, and fragrance) have been commercialized and introduced to consumers. Specifically,

D. cneroum L. (Rose daphne or garland flower) has become one of the most popular perennial flowering shrubs among discriminating ornamental plant growers. However, due to poor long-term performance of this and other daphne introductions, the genus has acquired a poor horticultural reputation. One of the major limitations to survival in cultivation is due to Daphne Sudden Death Syndrome (DSDS), a disease caused by the fungal root pathogen *Thielaviopsis basicola* (Berk. & Br.) Ferraris (syn. *Chalara elegans* Nag Raj et Kendrick) (Noshad et al. 2006*a*). This disease kills plants quickly, as the name suggests, following the first foliar symptoms. Observations on DSDS-infected plants indicate the following progression of symptoms: (i) brown to black necrotic lesions on the roots, (ii) leaf chlorosis leading to abscission, (iii) whole plant stunting, and (iv) stem collapse and plant death (Noshad et al, 2006*b*).

Thielaviopsis basicola is a fungus common in both cultivated and non-cultivated soils (King and Presley, 1942; Adams and Papavizas, 1969; Nag Raj and Kendrick, 1975; Yarwood, 1981) and is generally considered a facultative parasite with the ability to parasitize a wide range of important agricultural hosts (Bottacin et al., 1994; Anderson and Welacky, 1988; Papavizas, 1968; Reddy and Patrick, 1989). However, little is known about the factors (e.g., cultural conditions, host plant genetics) that affect DSDS development beyond anecdotal observations and practices. Previously, I identified taxon differences among 32 *Daphne* species and cultivars for resistance to *Thielaviopsis basicola*, by both *in vitro*- and *in vivo*-based methods (Noshad et al., in press). Plant reactions ranged from highly resistant, e.g., *D. tangutica* and *D. retusa*, to highly susceptible, e.g., *D. cneorum*.

The current study was guided by the following questions about the evolutionary relationships among selected *Daphne* taxa with reference to DSDS resistance: 1) 'Can the ITS region be an informative system for the phylogenetic analyses of *Daphne* species?'; 2) 'Is there any relationship between resistance against DSDS and the phylogenetic placement of *Daphne* species based on the ITS sequence analysis?'; 3) 'Can a pattern be identified that places the origin of disease resistance among *Daphne* species?'.

5.2 Material and Methods

5.2.1 Plant materials

All plant samples were taken from living plants maintained at the UBC Botanical Garden and Centre for Plant Research (UBGCPR). For all taxa, voucher specimens were prepared and deposited in the UBGCPR herbarium while photographs of flowering specimens were taken and deposited in the UBGCPR library. Twenty *Daphne* taxa (i.e., ingroup taxa) used in the phylogeny construction are listed with their region of nativity or origin and mean plant disease index (PDI) values (Noshad, 2006*b*) (Table 5.1). To investigate the relationship between *Daphne* taxa, appropriate outgroup selection is critical. The outgroup taxa should be systematically close enough to the species under observation to allow sequence alignment and yet distant enough to enable unequivocal rooting of the tree. Therefore, ITS1 and ITS2 sequences of 5 outgroup species i.e., *Dias cotinifolia* L., *Gnidia denudata* Lindi., *Pimelea spectabilis* Lindi., *Edgeworthia chrysantha* Lindi., and *Thymelaea hirsute* L. from the family Thymeleaeceae were obtained from NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) and used in the analyses.

5.2.2 DNA extraction and sequence data

Total genomic DNA was extracted from fresh samples collected from plants at UBC Botanical Garden. DNA isolation, PCR conditions, cycle sequence, and automated DNA sequencing were as specified in Moller and Cronk (1997). DNA sequence data were collected using the ABI Prism Dye Terminator Cycle Sequencing Kit (ABI PRISM. ®) and visualized using an ABI model Prism-377 DNA automated sequencer at the University of British Columbia NAPS (Nucleic Acid Protein Service). DNA was sequenced in both directions. Raw sequence data were imported into Sequencher 4.1® (Gene Codes Corporation, MI, USA), edited, and combined into a consensus sequence. Consensus sequences were imported into Se-Al® v 1.0 (Rambaut, 1996) and manually aligned using sequential pairwise comparisons with relative ease. Sequence length, alignment, and number of informative characters were determined using PAUP v. 4.0b10® (Swofford, 2000) and MacClade® (Maddison and Maddison, 2000). Summary data for these features are provided (Table 5.2).

5.2.3 Phylogenetic reconstruction

Phylogenetic analyses were performed using maximum parsimony (MP). Parsimony analysis was conducted assuming unordered character states and equal character weighting. Gaps were treated as missing data and these were excluded from all analyses. Parsimony analyses were completed using PAUP® v. 4.0b10 and employed a heuristic search strategy with 100 random stepwise-addition replicates, TBR branch swapping, and MULTREES optimization. Consistency index (CI; excluding uninformative characters) and retention index (RI) were also calculated (Farris, 1989; Kluge and Farris, 1969). Branch support was

determined with bootstrapping (Felsenstein, 1985) using a simple addition sequence and 10,000 replicates, with all other parameters equal to those used in the MP analysis. This analysis was followed by an exhaustive run with sample frequency (i.e., number of trees saved per generation) set at 20,000. The remaining trees from this analysis were saved and a consensus tree with posterior probability values was generated. Resistance patterns were examined by mapping PDI values (i.e., degree of resistance) onto the phylogeny using MacClade® v. 4.0. Ten classes of resistance were used as character states, based on statistical analysis of PDI values (Noshad, 2006*b*), for assessing the evolution of resistance (Fig 5.2).

5.3 Results

5.3.1 DNA matrix features and sequence divergence

Alignment of internal transcribed spacer sequences of the 25 taxa analyzed (Fig 5.1) resulted in a 610-bp long data matrix; the number of gaps after alignment was 166. The G+C content ranged from 48.3–65.4%. Sequence divergence values among the 25 taxa ranged from 31.8 to 49.6% with divergence among the ingroup taxa between 0.0–30.1%. Sequence characteristics are summarized (Table 5.2).

5.3.2 Phylogenetic results

The number of variable sites (excluding indels) detected in the entire ITS region was 245 (Table 5.2). Of these sites, 176 were phylogenetically informative. Parsimony analysis of unambiguously aligned ITS sequences yielded 63 most parsimonious trees (670 steps) with uninformative and gapped characters excluded. From the 63 most parsimonious trees, the

strict consensus tree is shown (Fig 5.1). Bootstrap and jackknife support values were similar. The topology of all parsimonious trees was nearly identical with respect to the inter-relationships among *Daphne* taxa. The strict consensus tree supports these selected *Daphne* taxa as a monophyletic group.

5.4 Discussion

The phylogenetic trees constructed give insight to the interrelationships among *Daphne* taxa. The most parsimonious trees suggest that *Daphne* is a monophyletic group (Fig 5.1). However, the placement for *D. genkwa* was unresolved and needs further research to more reliably place it in the phylogeny. Analysis of ITS sequence data resulted in a parsimony consensus tree with two well-supported clades and one clade with a lower level of support. Clade I (including *D. pontica, D. laureola, D. retusa. D. tangutica* and *D. longilobata*) had a bootstrap value of 100 and was comprised of species mostly from China but also includes species from N. Africa-middle east and Russia (Fig 5.2, Table 5.1). Clade II (including *D. oleoides, D. alpina, D. kosanini* and *D. cneorum*) had a bootstrap value of 100 and was comprised of species mainly from Europe. Clade III (including *D. giraldii, D. odora, D. bholua, D. gnidium, D. arbuscula, D. jasminea, D. caucasica, D. collina* and *D. circassica*) had the lowest bootstrap value (i.e., 78) and included species from around the world. In addition, there are two species (*D. genkwa* and *D. mezereum*) that are placed on separate branches from the other clades in all trees constructed (Fig 5.1). When country of origin is mapped to the consensus tree, no clear pattern emerges based on clade structure. Based on these data and the limited taxon sample, establishment of a clear pattern for nativity is difficult. When degree of resistance for individual species is mapped onto the consensus

parsimony tree, no clear pattern for the evolution of resistance is apparent. However, when many random trees were constructed based on degree of resistance, all were similar in structure. This step tested the null hypothesis of no structure, and confirms that the consensus tree produced is not a result of randomness. In addition, when the average PDI values of the species within the clade are compared (i.e., Clade I=23, Clade II=28, and Clade III=27), no significant differences were identified. However, despite these close PDI averages among the clades, sufficient structure is visible to propose a hypothesis on the evolution of disease resistance in *Daphne* (Fig 5.2). I hypothesize that resistance is a derived character and that plants recently evolved this ability. I base this hypothesis on presence of a higher number of susceptible species than resistant species in Clades II and III (Fig 5.2). Initially, the predecessor of modern *Daphne* may have been 'susceptible' due to a lack of selection pressure in its region of origin. However, after diversifying and colonizing new regions, they may have been exposed to the pathogen and evolved traits related to resistance. Additional taxon sampling will help identify the extent of resistance within this genus and perhaps identify its origin.

In addition, a hypothesis can be put forward that relates susceptibly of the species currently in horticulture production and the evolution of resistance in the genus. The pathogen is reported to be native to various parts of Asia preferring colder environments (Paulin-Mahady etal, 1994; Punja and Sun, 1999; Hood and Shew, 1997; Walbot, 1985; Wendel, 1992) while most of the horticulturally relevant species are native to the more moderate climates of Western European and Mediterranean regions. Therefore, when these species were grown in Canada, it may have been the first encounter with this pathogen in an environment allowing

for disease development. Because these plants may not be exposed to this pathogen in their native region, they would not have evolved the necessary traits for resistance and therefore display a high level of susceptibility.

The present study has limitations that future studies should address. One major limitation was the limited number of *Daphne* species sampled. While all accessible taxa were included, many additional taxa are described in the literature and need to be collected and included in future work. Inclusion of these additional taxa will help researchers develop a better understanding of *Daphne* evolution, and their evolving interaction with the pathogen. Another limitation was the use of a single isolate of pathogen in the germplasm screen. As other races of the pathogen may affect the disease ratings calculated for each taxon, subsequent changes in tree structure may occur. Therefore, collecting and using additional races of pathogen, from around the world, would help resolve this issue.

Based on the data collected from this research, I cannot establish a reliable pattern for the evolution of disease resistance for this genus. However, the results indicate that rDNA ITS sequences can be used for phylogenetic study at the sub-generic level in *Daphne*. Future research should target all other *Daphne* taxa, both in terms of disease assessment and molecular study, in order to clearly establish a valid and robust phylogeny. Once this phylogeny is complete, a more clear understanding of how resistance has evolved in this group may be possible.

5.5 References

Adams PB and Papavizas GC (1969) Survival of root-infecting fungi in soil. Phytopathology. 59, 135-138.

Alvarez I and Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference, Mol. Phylogen. Evol. 29, 417–434.

Anderson TR and Welacky TW (1988) Populations of *Thielaviopsis basicola* in burley tobacco field soils and the relationship between soil inoculum concentration and severity of disease on tobacco and soybean seedlings. Can. J. Plant Pathol. 10, 246-251.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS and Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. 82, 247–277.

Bottacin AM, Lévesque CA, and Punja ZK (1994) Characterization of dsRNA in *Chalara elegans* and effects on growth and virulence. Phytopathology. 84, 303-312.

Farris JS (1989) The retention index and homoplasy excess. System. Zool. 38, 406–407.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evol. 39, 783–791.

Halda J. J (2001) The Genus *Daphne*. Eva Kucerova- Sen Dobre Publikaca, Prague, Czeck Republic.

Hammond-Kosack KE, Jones JDG (1996) Disease resistance gene-dependent plant defense mechanisms. Plant Cell 8, 1773–91.

Hood ME and Shew HD (1997) Reassessment of the role of saprophytic activity in the ecology of *Thielaviopsis basicola*. Phytopathology. 87, 1214-1219.

Karban R and Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press, Chicago.

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions. Ann. Rev. Genetics. 24, 447–63.

Kim KJ, Jansen RK (1994) Comparisons of phylogenetic hypothesis among different data sets in dwarf dandelions (Krigia): Additional information from internal transcribe spacer sequences of nuclear ribosomal DNA. Plant Syst. Evol. 190, 157–185.

King CJ and Presley JT (1942) A root rot of cotton caused by *Thielaviopsis basicola*. Phytopathology. 32, 752-761.

Kluge AG, Farris JS (1969) Quantitative phyletics and the evolution of anurans. Syst. Zool. 18, 1–32.

Maddison WP, Maddison DR (2000) MacClade: Analysis of phylogeny and character evolution, Version 3.08. Sinauer, Sunderland, MA.

Mathew B, Brickell C and White R (2000) The smaller Daphnes. The Proceeding of the Conference " Daphne 2000 " April 2000. The Royal Horticultural Society, London.

Möller M and Cronk QC (1997) Origin and relationships of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA internal transcribed spacer (ITS) sequences. Amer. J. Bot. 84, 956–965.

Nag Raj TR and Kendrick B (1975) A monograph of *Chalara* and allied genera. Wilfred Laurier University Press, Waterloo, Ontario.

Noshad D, Punja ZK, Riseman A (2006*a*) First report of *Thielaviopsis basicola* on *Daphne* L.. Can. J. Plant Pathol. 28, 160-164.

Noshad D, Punja ZK and Riseman A (2006*b*) Screening for Resistance to *Thielaviopsis basicola* in Daphne spp. (2006) Can. J. Plant Pathol. 28, 310-312.

Papavizas GC (1968) Survival of root-infecting fungi in soil. Phytopathology. 58, 421-428.

Paulin-Mahady AE, Harrington TC and McNew D (2002) Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with Ceratocystis. Mycology. 94: 62-72.

Punja ZK and Sun LJ (1999) Morphological and molecular characterization of *Chalara elegans* (*Thielaviopsis basicola*), cause of black root rot on diverse plant species. Can. J. Bot. 77, 1801-1812.

Rambaut A (1996) Se-Al; Sequence Alignment Editor, ver. 1.0 alpha 1. Computer software available on the WWW site: htttp://evolve.zoo.ox.ac.uk/Se-Al.html.

Reddy MS and Patrick ZA (1989) Effects of host, nonhost, and fallow soil on populations of *Thielaviopsis basicola* and severity of black root rot. Can. J. Plant Pathol. 11, 68-74.

Swofford DL (2000) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0 Sinauer Associates, Sunderland, Massachusetts.

Todd E, Richter L, and Pamela C (2000) The evolution of disease resistance genes. Plant Mol. Biol. 42, 195–204.

Walbot V (1985) On the life strategies of plants and animals. Trends in Genetics. 1, 165–69.

Wendel JF and Albert VA (1992) Phylogenetics of the cotton genus (*Gosspium*): character-state weighted parsimony analysis of chloroplast-DNA restriction site data and its systematic and biogeographic implications. Systematic Botany 17, 115-143.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Amplifications. Academic Press, San Diego, p. 315–322.

Yarwood CE (1981) Occurrence of *Chalara elegans*. Mycology. 73, 524-530.

Table 5.1. *Daphne* taxa used in the *Thielaviopsis basicola* resistance bioassay with region of nativity or origin and mean plant disease index (PDI) values. Z: PDIs marked with the same letter(s) do not differ significantly, Tukey ($P<0.05$).

Taxa	Nativity/Origin	Mean PDI (SD) [z]
D. alpina L.	Italy	12.5 (0.92) [ghi]
D. arbuscula Celak.	Czech Republic	43.3 (1.74) [abcdef]
D. bholua Buch-Ham. ex	Nepal	12.5 (0.82) [ghi]
D. caucasica Pall.	Russia	9.2 (1.02) [hi]
D. circassica L.	Russia	46.6 (1.66) [abcde]
D. cneorum L.	Czeck republic	64.2 (1.89) [a]
D. collina Smith	Turkey	51.7 (1.31) [abcd]
D. genkwa Siebold & Zucc.	China	43.3 (1.31) [abcdef]
D. gnidium L	Spain	27.5 (1.22) [defgh]
D. giraldii Nitsche	W China	15.8 (1.18) [fghi]
D. jasminea Sibth.& Sm.	Greece	13.3 (1.09) [fghi]
D. kosaninii Stoj.	Bulgaria	20.8 (1.3) [efghi]
D. laureola L.	N Africa	23.3 (1.34) [defghi]
D. longilobata Turril.	China-Yunnan	30.8 (1.06) [cdefgh]
D. mezereum L.	Russia	10.0 (1.02) [hi]
D. odora Thunb.	China	31.7 (1.21) [cdefgh]
D. oleoides Schreber	Turkey	17.5 (1.19) [fghi]
D. pontica L.	Russia	60.8 (1.54) [ab]
D. retusa Hemsl.	China	0.0 (0) [j]
D. tangutica Maxim.	China	0.0 (0) [j]

Table 5.2. ITS sequence characteristics.

Total number of characters	610 bp
The number of characters parsimony informative	176 bp
The best recovered parsimony trees	63
Length	670
CI (consistency Index)	0.712
RI (Retention Index)	0.596

Figure 5.1. The consensus parsimony tree which is a strict consensus of the most parsimonious trees. values above branches represent bootstrap values (length: 670, CI: 0.712 and RI: 0.596).

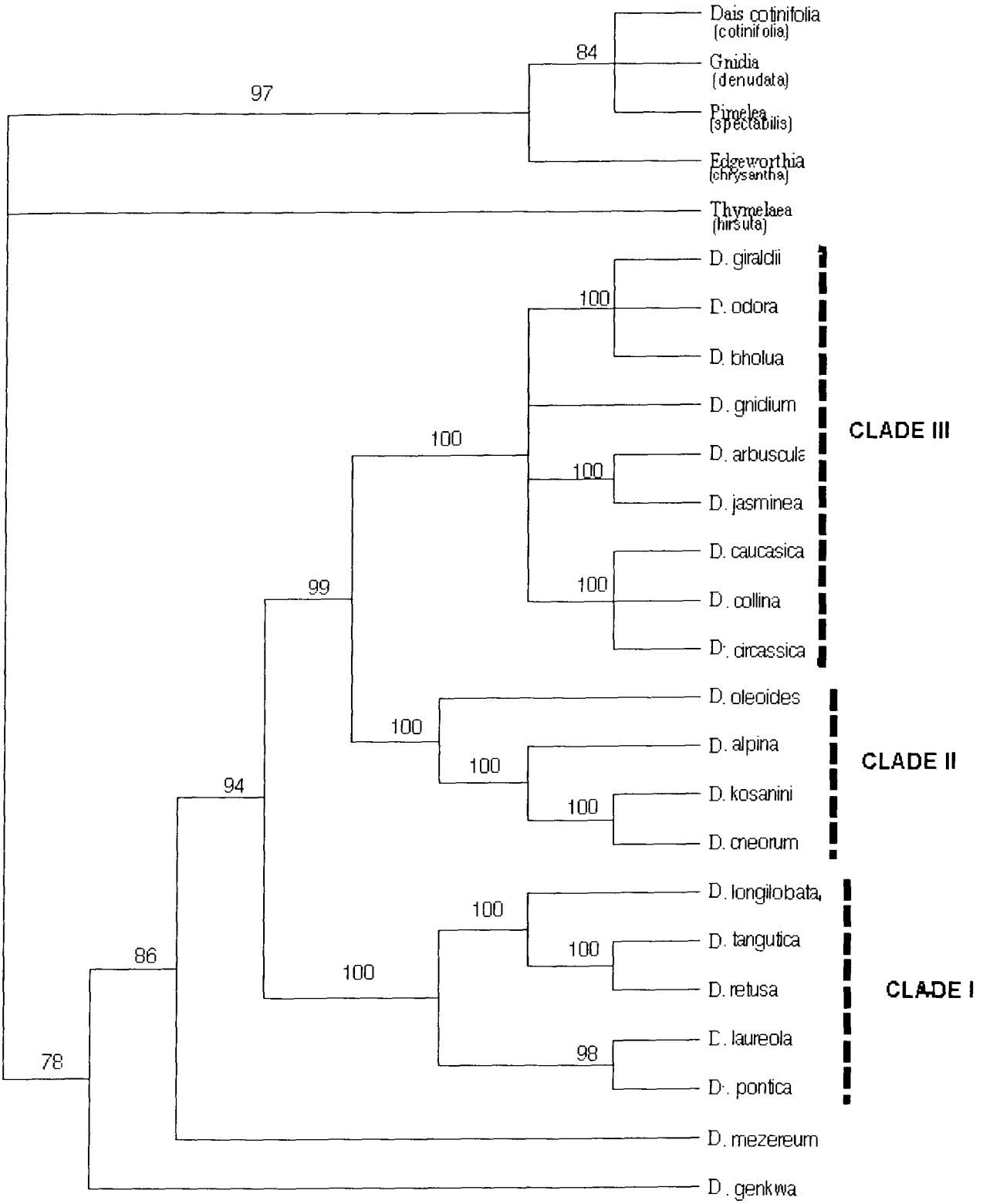
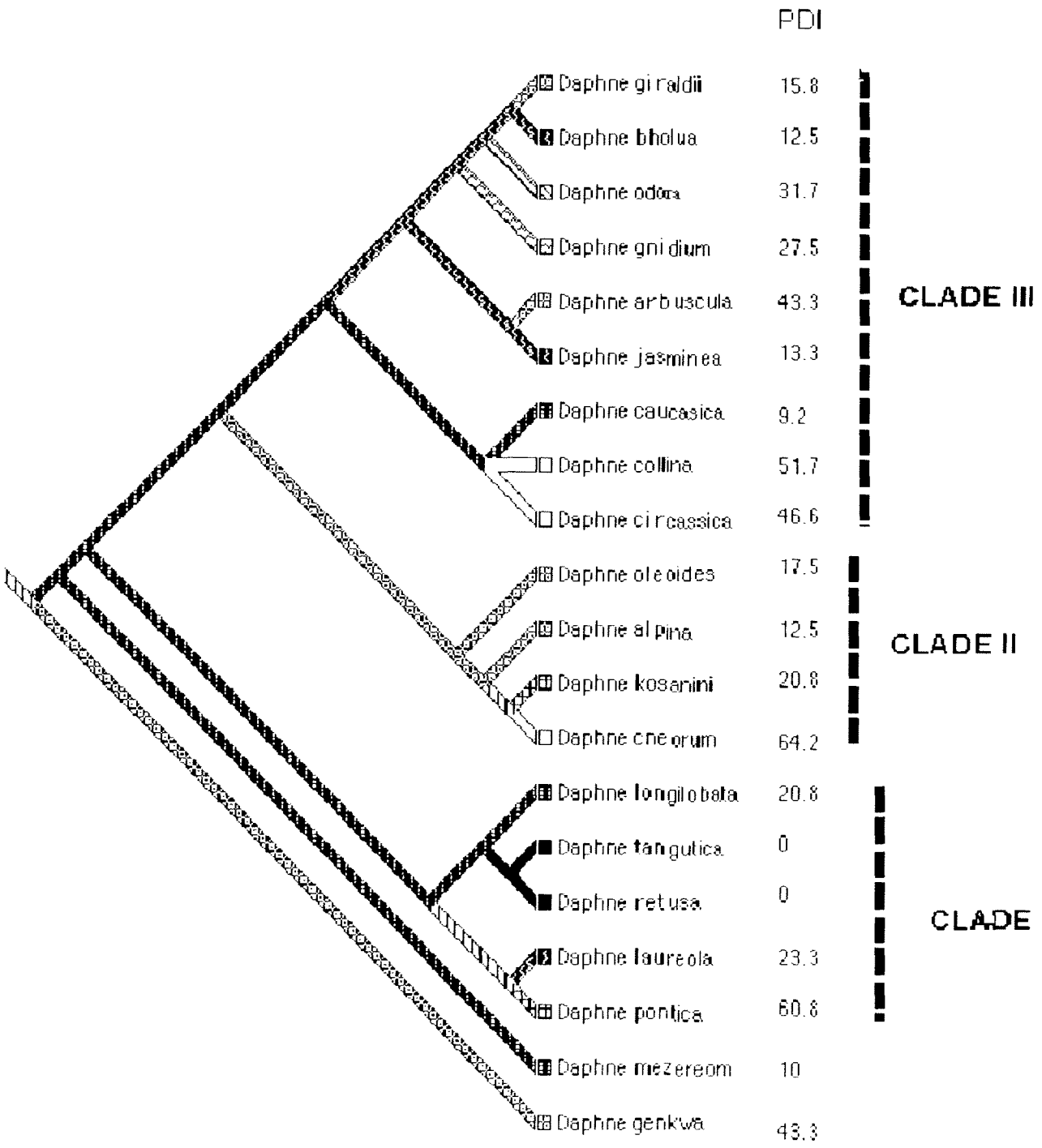


Figure 5.2. The evolutionary tree of resistance for the selected *Daphne* species. Solid branches and darker points indicate lineages for which resistance is stronger. PDI (Plant Disease Index) is shown next to each species. Three major clades are shown in the tree. The placement of *D. genkwa* is not completely clear and the ancestral trait is equivocal and shown by the striped pattern (▤).



CHAPTER SIX

CONCLUSIONS

6.1 Conclusions and Future Directions

Daphne is a widely dispersed genus with large variation in morphology, native habitats, and use. Despite their current ornamental, economic, pharmaceutical (Brickell and White, 2000), and ethnobotanical importance (Zhou, 1991), very little information is available about its pathology, phylogeny, or plant improvement techniques (e.g., biotechnology) (Marks and Simpson, 2000; Green etal, 1992; Cohen, 1977). In horticulture, broader acceptance of *Daphne* has been limited due to their susceptibility to Daphne Sudden Death Syndrome (DSDS). The major objective of the current research was to identify the causal agent for DSDS and to study variation in resistance among a sub-set of taxa. I used both in vivo and in vitro methods to screen selected *Daphne* taxa for resistance against DSDS. I also constructed a limited phylogeny of the selected *Daphne* species using rDNA ITS sequences and mapped resistance values onto the consensus parsimony tree. My analysis of resistance among taxa and their phylogenetic relationships increased our knowledge about this genus.

In Chapter 2, my observations documented the following progression of symptoms for DSDS: 1) brown to black necrotic lesions on the roots, 2) leaf chlorosis leading to abscission, 3) whole plant stunting, and 4) stem collapse and plant death. Though this research, I identified *Thielaviopsis basicola* (Berk. et BR.) Ferr. as the causal agent for this disease (Noshad, 2006a). In addition, a proven protocol for producing pure cultures of *T. basicola*

has been developed. These protocols were then used in the subsequent germplasm screen (Chapter 4).

In Chapter 3, I established species-specific protocols for *in vitro* propagation of the selected species for use in the *in vitro* disease evaluation (Noshad, 2006b). These protocols allowed for the successful production of uniform, disease-free plants for my experimental use. The results of the *in vitro* propagation indicated a pattern of respond to basal media. The two small shrub species of *Daphne* (*Daphne jasminea* and *Daphne cneorum*) responded best to WPM base media while the larger species responded best to MS based media. A more clear pattern might be identified in the future when *in vitro* propagation protocols are developed for additional species.

In Chapter 4, I evaluated the selected *Daphne* species for resistance against *T. basicola* under both *in vivo* and *in vitro* conditions by developing a disease progression rating (DPR). A plant disease index (PDI) was then developed based on both disease incidence and disease severity. Both *in vitro* and *in vivo* methods produced similar results and displayed a strong correlation. However, there was one notable difference between them for the rate of disease progression. Typically, the *in vitro* challenge produced a comparable level of disease as the *in vivo* challenge but in two to three weeks less time. For both methods, the inoculation and screening methods were proven robust in terms of the isolate's pathogenicity, disease characterization, consistency over time, and its ability to differentiate taxa. Based on the one isolate used, overall pathenogenicity was adequate in allowing assessment of disease incidence and severity (i.e., root and foliar symptoms) on all taxa. Also, the concentration of

conidia used was sufficient to cause disease but not to overwhelm the defense mechanism(s) and prevent taxa differences from being displayed. Based on the germplasm screens, significant differences among taxa were identified for resistance and ranged from highly susceptible (e.g. *D. cneroum*) to highly resistant (e.g. *D. tangutica* and *D. retusa*).

Finally, in Chapter 5, I constructed a limited phylogeny of selected *Daphne* taxa based on rDNA ITS sequences. I then associated this phylogeny with individual species' resistance against *T. basicola*. Using established phylogenetic analysis to construct a consensus parsimony tree, *Daphne* appears to be a monophyletic group. However, placement of *D. genkwa* remains problematic and needs to be studied further. Analysis of ITS sequences resulted in a parsimony consensus tree with two well-supported major clades and one clade with a lower level of support. The tree for resistance evolution inferred from the phylogenetic data and the results of the germplasm screen are not sufficiently clear to make conclusions regarding the evolution of disease resistance. I propose that resistance is a new characteristic and these plants evolved resistance against the pathogen after colonizing new regions. This hypothesis is supported by the present of more susceptible species than resistant species among the examined group. By studying additional taxa of this genus, we may be better able to relate phylogenetic relationships among *Daphne* taxa with respect to the evolution of disease resistance.

Many questions remain for future research and I strongly hope that these results will be of significant assistance to those future researchers. Some of the major questions remaining include "How do other *Daphne* species interact with *T. basicola*?", "What are the

phylogenetic relationships among the whole of *Daphne*?", and finally " How would other races of *T. basicola* react with these tested *Daphne* as well as other species?". Basically, every disease is a product of interactions between three major factors, the host, the pathogen, and the environment surrounding them. In my study I tried to better understand DSDS and the major issues surrounding its basis and development. Although I was able to identify the pathogen responsible and some of its characteristics, more details are needed on its interaction with other local microorganisms, its infection and penetration methods and its interaction with resistant/susceptible plants. Since many aspects of *Daphne*'s anatomy and physiology remain unknown, but may be related to disease resistance, future research should try to identify and characterize these underlying traits. Also, the environments in which I conducted this project were limited and did not assess disease development under a wide range of conditions. Future research should also evaluate the environmental effects on DSDS development, its influence on *Daphne* physiology, and its interaction with the pathogen.

6.2 References

Brickell CD and White R (2000) A quartet of New Daphnes. The New Plantsman. 7, 6-18. The Royal Horticultural Society, London.

Cohen D (1977) Thermotherapy and meristem-tip culture of some ornamental plants. Acta Hort. 78, 381-388.

Green MJ, Godkin SE and Monette PL (1992) Use of in vitro cultures of *Daphne cneorum* L. for the western detection of daphne virus X. J. Enviro. Hort. 10(3), 153-155.

Marks T and Simpson S (2000) Interaction of explant type and indol-3-butyric acid during rooting in vitro in a range of difficult and easy to root woody plants. Plant Cell, Tiss. Org. Cult. 62, 65-74.

Noshad D, Punja ZK, Riseman A (2006*a*) First report of *Thielaviopsis basicola* on *Daphne* L. Can. J. Plant Pathol. 28, 160-164.

Noshad D, Punja ZK and Riseman A (2006*b*) Screening for Resistance to *Thielaviopsis basicola* in *Daphne* spp. (2006) Can. J. Plant Pathol. 28, 310-312.

Zhou B (1991) Some progress on the chemistry of natural bioactive terpenoids from Chinese medicinal plants. Memorias do instituto Oswaldo Cruz. 86 (suppl. 2) 219-226.

Appendix 1 – Preliminary Experiments on Inoculation Protocol Development

Introduction

Preliminary experiments were conducted to determine the appropriate concentration of pathogen spores needed for effective inoculation of *Daphne* species. The main objective of this experiment was to determine how many spores were sufficient to induce disease in the susceptible species, *D. cneorum*.

Material and Methods

A single-spore derived, aggressive isolate of *T. basicola,* isolated from diseased daphne plants, was used throughout this study. A suspension of endoconidia was prepared by gently washing the surface of 3-week-old colonies with deionized water and vortexing the wash solution for 30s. The resulting suspension was twice filtered through four layers of cheesecloth to remove agar, hyphae, and chlamydospores. Prior to inoculation, spore suspensions were calibrated with a haemocytometer and adjusted with deionized water to obtain endoconidia concentrations of $1x10^4$ ml^{-1}, $1x10^5$ ml^{-1}, $1x10^6$ ml^{-1}, and $1x10^7$ ml^{-1}.

Rooted plants of *Daphne cneorum* were produced in July and August from terminal cuttings (50-100 mm in length) with the flower buds and lower leaves removed. Cuttings were made with a single shallow cut and soaked in an anti-fungal solution (Physan 20, Maril Products Inc.) for 60 seconds. The cuttings were allowed to dry momentarily before being dipped in 0.4% IBA powder (Stim Root #2, Plant Products. Co. Ltd.) and then placed in 6 cm pots

filled with a course rooting medium (10 parts propagation grade perlite, 8 parts peat, 6 parts granite grit #2, 1 part pumice (double screened to remove fine particles), dolomite lime 65AG at 900 g m^{-3}, and Micromax (trace elements) at 400 g m^{-3}). The flats were placed under intermittent mist with bottom heat set at 22°C. Rooted cuttings were transferred to a polyhouse in October where they were allowed to go dormant but kept frost free. They were repotted in May into 12 cm pots filled with a well-drained medium (8 parts peat, 8 parts Turface MVP, 6 parts granite grit #2, 4 parts screened and pasteurized soil, 1 part pumice, dolomite lime 65AG at 670 g m^{-3}, Micromax micronutrients at 540 g m^{-3}, Osmocote 18-6-12 at 2150 g m^{-3}, and Psi Matric wetting agent). All stock plants were grown under shade cloth during the summer months and moved to a heated polyhouse during the winter months to prevent frost damage. Fertilization regiment included yearly top-dressing with Osmocote 18-6-12 at 5 g 1 gal pot. Fungicides were not used during stock production because we had very little disease pressure and we did not want to risk cross-contamination affecting the in vivo assay.

Innoculation procedures consisted of topically applying 1 ml suspension to healthy roots of 2-year old nursery-grown *D. cneorum* plants. Control plants were treated with sterilized distilled water. Production containers were modified to contain a clear panel behind a lightproof 'door' to allow for direct observation of the infection process without further disturbance to the root system. To allow for uniform conditions following inoculation, plants were transferred to a greenhouse and grown under natural light at 24 +1 °C and a relative humidity between 70 and 80%. In this experiment, a completely randomized design

used with three replications. Each replication consisted of 5 plants with two plants serving as controls.

Results

The results of this preliminary experiment indicated that a minimum of $1x10^6$ spores were needed for regular and reliable infection. At this concentration, 100% of the treated plants displayed symptoms within four weeks (Table 1). Concentrations below this amount failed to induce disease on 100% of the treated plants. Concentrations above $1x10^6$ also induced disease on 100% of the plants but within the same time period as $1x10^6$. Therefore, I used this concentration for all disease assessment trials.

Table 1- Percent diseased plants 4 weeks following inoculation.

#	Spore concentration per ml	Diseased Plants (%)
1	$1x10^4$	6.66
2	$1x10^5$	46.66
3	$1x10^6$	100
4	$1x10^7$	100

Appendix 2 –Experiments on PGR Stability Following Autoclaving

Introduction

Experiments were conducted to evaluate the stability of the PGRs used in this research following autoclaving. Manufacturer specifications indicate that IAA, IBA, and KIN may have reduced activities following autoclaving while NAA is reported to be stable. Specifically, I compared shoot production on *D. cnerourm* following treatments with PGRs that were either autoclaved (121 C for 15 min) or filtered sterilized.

Materials and Methods

Previously established cultures of *D. cneorum* growing on multiplication medium (Chapter 3) yielded shoots used in these trials. For the trials, fresh multiplication media were prepared as described previously but with PGR supplements either added to the medium before autoclaving (121 C for 15 min) or added following autoclaving and filter sterilization. Each treatment had 12 subsamples. Paired t-tests were performed comparing shoot production from autoclave vs. filtered sterilized within a medium.

Results

Average number of new shoots produced 4 weeks following culture are listed in Table 1. In four of the five PGR combinations, no difference in shoot production was observed between autoclaved vs. filtered PGRs. However, in the 2 mg l^{-1} BA + 0.01 mg l^{-1} IBA treatment, a significant difference was detected. The autoclaved treatment produced more shoots than its

filtered counterpart. In general, these data are similar in range and magnitude of those data detailed in Chapter 3. However, in three of the five treatments, current averages are slightly lower than those previously reported. I attribute this difference to the extended evaluation time used in Chapter 3. In the current study, the data were collected at four weeks; not the eight weeks used in Chapter 3. These four additional weeks of growth may have allowed the cultures to produce more shoots than what is currently observed. Based on these results, I conclude that under the autoclave conditions used (121 C for 15 min), no reduction in PGR activity was observed and that the procedures used (i.e., autoclaving the PGR supplements) did not affect my results or conclusions.

Table 1. Shoot production of *D. cneorum* after four weeks of culture on one of the listed media. PGR supplements were either autoclaved with all other medium components or added after autoclaving and filter sterilization.

PGR Supplement	Mean Shoot Production (SD) Autoclaved	Mean Shoot Production (SD) Filtered	*P* Two-tailed t-test	Chapter 3 Means
1 mg l^{-1} BA + 0.1 mg l^{-1} IAA	3.3 (1.2)	3.2 (0.9)	0.72	2.2 (0.6)
2 mg l^{-1} BA + 0.01 mg l^{-1} IBA	4.9 (1.1)	3.5 (1.4)	0.03	3.9 (0.9)
2 mg l^{-1} BA + 0.01 mg l^{-1} NAA	3.1 (1.7)	3 (1.3)	0.73	5.6 (0.7)
4 mg l^{-1} KIN + 0.01 IAA mg l^{-1}	2.7 (0.9)	2.2 (1.2)	0.17	2.7 (0.8)
0.001 mg l^{-1} TDZ + 0.01 mg l^{-1} IAA	1.2 (0.9)	1.4 (0.9)	0.67	2.8 (0.9)

Appendix 3 – Preliminary Experiments on *in vitro* Propagation Media Composition

Introduction

Preliminary experiments were conducted to evaluate the effects of modifying the concentrations of base medium components on growth of selected *Daphne* species. The specific objective was to evaluate whether changes (mostly reductions) in concentrations of the base salts, sugar (carbon source) and gelling agent (agar) affected shoot and root growth of *Daphne* plantlets.

Material and Methods

I. Plant material

In preliminary experiments, I used the following seven species *D. cneorum*, *D. caucasica*, *D. retusa*, *D. giraldii*, *D. jasminea*, *D. laureola* and *D. tangutica*.

II. General procedures

Apical shoot tips, 2-5 cm in length, 3-5 mm in diameter, and bearing 1-3 nodes were collected during the summer and fall seasons from 4-6 year old container-grown plants. Collected shoots were striped of all leaves and rinsed under running tap water for 15 min. Under aseptic conditions, shoots were then surface sanitized by treatment with 70% ETOH for 30 seconds followed by treatment with 0.5% sodium hypochlorite solution containing 0.5

ml l^{-1} Tween-20 (Sigma Chemical, St. Louis, MO) and gently stirred for 10 min. After sanitation, shoots were rinsed three times with sterile distilled water for 5 min each and placed individually in culture vessels. Cultures were maintained at 25°C under 16 h photoperiod with irradiance intensity of 350 $\mu W\ cm^{-2}$ supplied by cool white fluorescent lamps.

The pH of the various media was adjusted based on published protocols (e.g., MS media was adjusted to 5.8, WPM media to 5.2) before autoclaving for 15 min at 121°C. Explants were maintained on establishment media i.e. basic MS and WPM with 20 g l^{-1} sucrose, and 5.6 g l^{-1} high gel strength agar (Sigma-Aldrich, St. Louis, MO) without PGRs, for four weeks prior to subculture on multiplication media. Following a four week establishment period, shoots were sub-cultured into 150 mm baby food jars (Sigma-Aldrich, St. Louis, MO) containing 25 ml multiplication media. Basically, nodal explants cultured on either MS (Murashig and Skoog, 1962) or WPM (Woody Plant Medium; McCown and Lloyd, 1983) supplemented with plant growth regulators (i.e., 2 mg l^{-1} BA + 0.01 mg l^{-1} NAA), recommended minerals and 5.6 g l^{-1} agar with vitamins (nicotinic acid 0.5 g l^{-1}, pyridoxine-HCl 0.5 g l^{-1}, thiamine-HCl 0.1 g l^{-1}, glycine 2 g l^{-1}), based on these two major protocols (Murashige and Skoog, 1962; McCown and Lloyd, 1983).

Following multiplication, new shoots were used in elongation and rooting trials. For shoot elongation, shoots 1 cm long with intact apices and 2-4 leaves were cultured on basic media (either MS or WPM basal medium) supplemented with 1 g l^{-1} charcoal without PGRs. Following four weeks, the longest shoots (4-6 cm) were transferred to individual test tubes

with 20 ml media consisting of base MS or WPM components but modified as follows (no PGR has been used):

#	Media
1	Basic medium (BM)- without PGR
2	1/2 BM strength
3	1/4 BM strength
4	1/8 BM strength
5	BM + 2x sucrose (40 g l^{-1})
6	BM+ 1/2x sucrose(10 g l^{-1})
7	BM+ 1/4x sucrose (5 g l^{-1})
8	BM+ 1/8x sucrose (2.5 g l^{-1})
9	BM+ 2x gelling agent (11.2 g l^{-1} high gel strength agar)
10	BM+ 1/2x gelling agent (2.8 g l^{-1} high gel strength agar)
11	BM+ 1/4x gelling agent (1.4 g l^{-1} high gel strength agar)
12	BM+ 1/8x gelling agent (0.7 g l^{-1} high gel strength agar)

BM = Standard MS or WPM base salts and vitamins

All experiments used a complete randomized design with three replications and 15 shoots per replication (i.e., 45 shoots). Data were recorded as percentage of shoots rooted after 8 weeks.

Results

The results of these experiments indicated that none of the modifications evaluated improved shoot or root growth of any *Daphne* species. In addition, the most robust growth was observed from the originally published composition. Therefore, all *in vitro* propagation experiments used the standard base media (MS/WPM) compositions.

References

McCown BH and Lloyd GB (1983) A survey of the response of *Rhododendron* to *in vitro* culture. Plant Cell Tiss. Org. Cult. 2, 75–85.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 473–497.