# Optimizing short term conservation methods for a functionally extinct tree species, American chestnut (*Castanea dentata* (Marsh.) Borkh.)

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## ABSTRACT

Tissue culture is a powerful conservation tool, particularly in the preservation of species which have ongoing restrictions in seed production or which cannot be otherwise easily propagated. American chestnut (*Castanea dentata* (Marsh.) Borkh.) is functionally extinct in its native range. Some sub-populations are too isolated for seed production in this self-incompatible species, and *C. dentata* is recalcitrant to conventional clonal propagation. Short term conservation methods for *C. dentata* in tissue culture were developed and improved upon in this work. Methods to improve multiplication rate (physiological stage of source material and incubation temperature) and microshoot quality (effect of exogenous medium modifications in 6-benzylaminopurine: BAP, calcium, magnesium, boron, and gelling agent type and concentration on the incidence of shoot top necrosis (STN) were investigated in the axillary shoot culture of genotypes from diverse sub-populations of *C. dentata*. Physiological stage of source material affected multiplication rate, with seedling-stage source material producing the most vigorous cultures. Incubation temperature affected multiplication rate in three out of eight genotypes tested. The most concentrated BAP significantly reduced the incidence of STN. No other exogenous medium treatments reduced STN. A framework for developing a conservation plan utilizing axillary shoot culture with *C. dentata* was also discussed. A somatic embryogenesis protocol utilizing axillary shoot cultures as microcuttings was developed, with a highly successful embryogenesis production rate (14-19 out of 20 microcuttings in competent genotypes). As well, a review was conducted critically assessing known information on the metabolomics of the host-pathogen interaction between *Cryphonectria parasitica* and *Castanea* spp. These tissue conservation tools and metabolomic insights will allow for the more effective preservation of this species for future generations.

## RÉSUMÉ

La culture tissulaire est un puissant outil de conservation, en particulier pour la préservation des espèces soumises à des restrictions permanentes dans la production de semences, ou qui ne peuvent pas être facilement reproduites. Le châtaignier d'Amérique (*Castanea dentata* (Marsh.) Borkh.) est fonctionnellement éteint dans son aire de répartition naturelle. Certaines sous-populations sont trop isolées pour la production de graines chez cette espèce auto-incompatible, et *C. dentata* est récalcitrant à la propagation clonale conventionnelle. Des méthodes de conservation à court terme pour *C. dentata* en culture tissulaire ont été développées et améliorées dans ce travail. Méthodes permettant d'améliorer le taux de multiplication (stade physiologique du matériau source, et la température d'incubation) et la qualité du micro-pied (effet des modifications du milieu exogène sur la 6-benzylaminopurine: BAP, calcium, magnésium, bore, et agent gélifiant (et concentration) sur l'incidence de nécrose au sommet de la tige (STN) ont été étudiés dans la culture axillaire de génotypes de diverses sous-populations de *C. dentata*. Le stade physiologique de la matière source a entraîné un taux de multiplication, la matière source au stade des semis produisant les cultures les plus vigoureuses. La température d'incubation a eu une incidence sur le taux de multiplication de trois des huit génotypes testés. La BAP la plus concentrée a réduit de manière significative l'incidence de STN. Aucun autre traitement avec un milieu exogène n'a réduit le STN. Un cadre pour l'élaboration d'un plan de conservation utilisant la culture de pousses axillaires avec *C. dentata* a également été discuté. Un protocole d'embryogenèse somatique utilisant des cultures de pousses axillaires comme micro-coupures a été développé, avec un taux de production d'embryons extrêmement réussi (14 à 19 sur 20 micro-coupes chez des génotypes compétents). En outre, une analyse critique de l'information connue sur la métabolomique de l'interaction hôte-pathogène entre *Cryphonectria parasitica* et *Castanea* spp.. Ces outils de conservation des tissus et ces informations métabolomiques permettront une préservation plus efficace de cette espèce pour les générations futures.

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## CONTRIBUTION OF AUTHORS

**Dr. Danielle J. Donnelly**, the candidate's supervisor, was a key motivator for the thesis. Dr. Donnelly provided input in the all study designs, and provided continual guidance and feedback during data collection. Dr. Donnelly also provided extensive feedback and input to all sections of this thesis and the associated manuscripts.

**Christie-Anna Lovat** (Candidate) was responsible for acquiring the source material of *C. dentata* for culture, networking within the TACF and CCC communities, and developing methodologies for all Stages of axillary shoot culture and somatic embryogenesis in *C. dentata*. The candidate prepared all media and performed all subcultures necessary for both research and regular culture maintenance. The candidate was also responsible for the maintenance of all plants growing ex vitro. The candidate collected all the data included in this thesis, conducted all statistical analyses, and developed all figures and tables for this thesis. The candidate wrote all the chapters of this thesis and the associated manuscripts.

## STATEMENT OF ORIGINALITY

### I. Claims of original research

The aim of this doctoral dissertation was to explore and improve upon short term conservation methods for *Castanea dentata*. In Study 1 (Chapter 3), axillary shoot culture was investigated as a conservation tool for *C. dentata* through a combination of two main objectives. The first aspect involved improving the multiplication rate of *C. dentata* in axillary shoot culture by identifying: (1) the effect of growth stage of the source material, (2) the effect of incubation temperature and isotherm of origin of the source material, (3) the effect of genotype on multiplication rate, and (4) optimizing a conservation framework at the genotype level. The second aspect involved reducing the incidence of shoot tip necrosis (STN) by examining the effect of several medium factors on *C. dentata* during axillary shoot culture. These included: (1) BAP, (2) calcium (Ca), boron (B), magnesium (Mg), and combinations of Ca/Mg and Ca/B, and (3) gelling agent type (agar and gellan gum) and concentration. In Study 1, all goals were achieved. Growth stage of the source material had a significant effect on multiplication rate. The most vigorous cultures were derived from the seedling-stage source material. Incubation temperature impacted STN across genotypes, and had a genotype-specific effect on multiplication rate, independent of the isotherm of the source material. Multiplication rate was significantly different across some, but not all, genotypes. Factors were identified which varied significantly at the genotype level (incubation temperature, overall multiplication rate), and those which were relatively similar across genotypes (response to changes in medium components). From this information, three separate axillary shoot culture systems were recommended for the axillary shoot culture of *C. dentata* from a conservation perspective, in order to maximize multiplication rate based on the phenotypic variability observed in this study. Translocation issues were determined to be the primary cause of STN in *C. dentata*, and the incidence of STN was reduced in the genotypes investigated.

In Study 2 (Chapter 4), the second short term conservation method, somatic embryogenesis, was explored in *C. dentata*. The main goal was to develop an efficient somatic embryogenesis method to: (1) increase the rate of Stage 1 induction of somatic embryogenesis in microcuttings above rates reported in the literature (0.02 – 3.00 %), (2) utilize axillary shoot culture tissues as explants for somatic embryogenesis, and (3) demonstrate that somatic embryogenesis is

possible with genotypes derived from mature-phase tissue explants. In Study 2, two of these three goals were achieved. A somatic embryogenesis procedure was developed utilizing single node microcuttings derived from axillary shoot cultures. Stage I induction rate of somatic embryogenesis was determined to be 70.0 – 95.0 % of microcuttings in two out of three genotypes tested. Both seedling-stage derived genotypes were capable of somatic embryo formation under this protocol. However, somatic embryos failed to form in the only mature-phase genotype examined. Somatic embryos produced in this work developed to the early torpedo stage.

In Study 3 (Chapter 5), metabolites were described which could have value in the development of *C dentata* with improved blight tolerance. This was accomplished by: (1) determining the known virulence factors from *Cryphonectria parasitica* in *Castanea* spp., (2) ranking known virulence factors based on their relative importance to virulence in the host-pathogen interaction (based on available literature), and (3) determining host defence factors. This review provides the first critical assessment of the metabolomics of the host-pathogen interaction between *C. parasitica* and its primary hosts, *Castanea* spp. Images of host tissues during active blight infection were described, among very few such images available in the past 100 yr and the first in juvenile individuals. These photos are the first to visually capture the presence of calcium oxalate crystals and intense tannins in host tissues surrounding mycelial mats during an active blight infection.

Taken together, the findings of this thesis demonstrate the utility of short term tissue culture conservation methods for the preservation of *C. dentata*, and provide the tools necessary to make such a conservation program a reality. The results of the axillary shoot culture component of this thesis will be immediately implemented by the CCC in the conservation of *C. dentata* in Southern Ontario as part of their "Breaking Isolation" provincial recovery plan, while the somatic embryogenesis work and metabolomics review will be immediately used by the American Chestnut Research and Restoration Project of SUNY-ESF as a part of their ongoing genetic transformation conservation program for *C. dentata*.

## II. Peer reviewed publications and submitted manuscripts

- **Lovat C-A**, Donnelly DJ (201X, Accepted March 2019) Optimizing axillary shoot culture as a short term conservation method for American chestnut (*Castanea dentata* (Marsh.) Borkh.). Can J For Res

- **Lovat C-A**, Donnelly DJ (201X, submitted Feb 2019) Advances in the indirect organogenesis of American chestnut (*Castanea dentata* (Marsh.) Borkh.) for short term conservation. Can J For Res
- **Lovat C-A**, Donnelly DJ (201X, Accepted March 2019) Mechanisms and metabolomics of the host-pathogen interactions between Chestnut (*Castanea* species) and Chestnut blight (*Cryphonectria parasitica*). Forest Pathol
- **Lovat C**, Nassar AM, Kubow S, Li XQ, Donnelly DJ. (2016) Metabolic biosynthesis of potato (*Solanum tuberosum* L.) antioxidants and implications for human health. Crit Rev Food Sci Nutr 56:2278-2303
- de Blois S, Boisvert-Marsh L, Schmucki R, **Lovat C-A**, Byun C, Gomez-Garcia P, Otfinowski R, Groeneveld E, Lavoie C. Outils pour évaluer les risques d'invasion biologique dans un context de changements climatiques. 2013. McGill University, Consortium Ouranos. http://www.ouranos.ca/media/publication/239_RapportdeBlois2013.pdf

**III. Conference abstracts and presentations**

- **Lovat C**, Donnelly DJ (2018) Saving the American chestnut in Canada. Paper presented at: Quebec Nut Growers Association and *club des producteurs de noix comestibles du Québec* Joint Conference; August 5-8; St-Paulin, QC
- **Lovat C**, Donnelly DJ (2017) Micropropagating elite Canadian *Castanea dentata*. Paper presented at: Canadian Chestnut Council Symposium; October 21; St. George, ON
- **Lovat C,** Donnelly DJ (2017) Restoration of the American chestnut (*Castanea dentata*) in Canada. Paper presented at: Quebec Society for the Protection of Plants Annual Conference; June 8-9; Ste-Anne-de-Bellevue, QC
- **Lovat C**, Donnelly DJ (2016) Tissue culture for the multiplication and improvement of American chestnut (*Castanea dentata* (Marsh.) Borkh.). Paper presented at: Canadian Chestnut Council Symposium; February 23; St. George, ON
- **Lovat C**, Donnelly DJ (2015) Past, present, and future of American chestnut. Paper presented at: Quebec Nut Growers Association Annual Meeting; November 7; Ste-Ambroise-de-Kildare, QC
- **Lovat C**, de Blois S (2015) *Phragmites australis* phenology along a climate gradient. Paper presented at: Society of Wetland Scientists Annual Meeting; May 31-June 4; Providence, RI

- **Lovat C**, Donnelly DJ (2014) Somatic embryogenesis as a breeding tool in American chestnut (*Castanea dentata* (Marsh.) Borkh.). Paper presented at: The 9th Canadian Plant Biotechnology Conference; May 12-15; Montreal, QC
- **Lovat C**, Donnelly DJ (2014). Somaclonal variation as a breeding tool in American chestnut. Paper presented at: Canadian Chestnut Council Symposium; March 26; St. George, ON

**IV. Popular publications**

- **Lovat C-A** (2015) A Canadian in Syracuse. The American Chestnut Foundation eSprout, August 2015
- **Lovat C-A** (2014) Trees in test tubes. Maryland Chapter of the American Chestnut Foundation Fall 2014 Newsletter

**V. Websites**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 2,4,5-T | 2,4,5-trichlorophenoxyacetic acid |
| 4-CPPU | N-(2-chloro-4-pyridyl)-N-phenylurea |
| ABA | abscisic acid |
| TACF | The American Chestnut Foundation |
| BAP | 6-benzylaminopurine |
| CAM | Chestnut Axillary Medium |
| CCC | Canadian Chestnut Council |
| CIP | culture-induced phenotype |
| DBH | diameter at breast height |
| EDTA | ethylene diamine tetra-acetate |
| EPA | Environmental Protection Agency |
| FDA | Food and Drug Administration |
| IBA | indole-3-butyric acid |
| $GA_3$ | gibberellic acid |
| GSPC | Global Strategy for Plant Conservation |
| HSP | heat shock protein |
| IAA | indole-3-acetic acid |
| K | Kinetin |
| MS | Murashige and Skoog |
| MYA | million years ago |
| NAA | naphthaleneacetic acid |
| NN | Nitsch and Nitsch |
| PBZ | Paclobutrazol |
| PCIB | 2-(4-chlorophenoxy)2-methylpropionic acid |
| RH | relative humidity |
| STN | shoot tip necrosis |
| SUNY-ESF | College of Environmental Science and Forestry of Syracuse University |
| TDZ | Thidiazuron |
| UN | United Nations |

| | |
|---|---|
| USDA | United States Department of Agriculture |
| VC | vegetative compatibility |
| WPM | Lloyd and McCown's Woody Plant |
| Z | zeatin |

# CHAPTER 1. Introduction

## 1.1 Rationale and statement of purpose

In the 21st century and beyond, the world is facing a staggering loss in biodiversity. It is estimated that 20.0 % of existing plant species will be extinct within this century (Brummitt et al. 2015). In this context, conservation has never been more relevant to plant ecology. The Global Strategy for Plant Conservation (GSPC) of the United Nations (UN) is an international organization which was created to address the issue of plant conservation. A goal of the GSPC is to conserve 75.0 % of threatened species through seed storage by 2020 (Wyse et al. 2018). However, not all plant species can be stored over the long term using the seed storage protocols practiced by the GSPC. For example, some species only increase vegetatively (Engelmann 2004). In some particularly rare species, which may now consist of a single specimen, seed production may no longer be possible in situ (Mao et al. 2018). For example, Wood's cycad (*Encephalartos woodii*) is known from a single male individual documented in 1895 (Prakash et al. 2008). Over 500 clones of this individual are currently found in botanical gardens internationally. A female plant has never been located, despite extensive searches, rendering this species incapable of seed production through sexual reproduction. As well, some seeds cannot be stored over the long term. Recalcitrant seeds, among them many tropical species, cannot maintain their viability when dried (Li and Pritchard 2009). Some species have a naturally short dormancy period (Mondoni et al. 2011). Alpine species have been shown on average to have a shorter seed viability period than closely related lowland species. As well, it may be desirable to preserve the germplasm of a select or elite individual of a species (Whittemore and Olsen 2011). Where a species is under intense selection pressure by an introduced pathogen, such as American elm (*Ulmus americana*) and *Ophiostoma* spp. (Dutch elm disease), conserving the genotypes of select individuals with apparent tolerance to the pathogen is a priority for conservation. However, if that species is self-incompatible or otherwise cannot produce seed with the same genotype as the parent, it would not be possible to preserve genotypes or select individuals through seed. Conservation through seed is also limited by the number of propagules naturally produced by the species (Van Drunen et al. 2017). For example, a rare species may only be able to produce a limited number of seeds in the extant population, a specialized obligate pollinator may be declining or extinct, or individuals may be dispersed widely enough that natural cross-pollination is no longer possible and seeds must be produced through manual hand-pollination (Philips et al. 2015; Van Drunen et al. 2017). Tissue

culture techniques have increasingly been used as conservation methods for species which cannot be easily conserved through traditional seed conservation methods.

Tissue culture methods for conservation have arisen from the needs of long term germplasm storage programs such as the GSPC (Corredoira et al. 2017). As such, methods are classified based on the length of time material is to be stored without human intervention. Short term conservation methods involve protocols which require weekly to monthly inputs, such as axillary shoot culture or somatic embryogenesis. Medium term preservation methods involve protocols which do not require inputs over months to a few years. Generally these include slow growth protocols of existing axillary shoot or somatic embryogenesis cultures. Long term methods involve storage over years to decades, and generally are limited to cryopreservation techniques (Corredoira et al. 2017).

Drastic population declines have already occurred in some species which cannot be easily conserved through seed storage (Mao et al. 2011). For these declining species, the rapid application of tissue culture conservation methods may be necessary to preserve them for future generations. One of the most emblematic of these declining plant species is the American chestnut (*Castanea dentata* (Marsh.) Borkh.) of North America (Anagnostakis 2012).

*C. dentata* was at one point a dominant overstory tree species in North America (Wang et al. 2013). In the early 20$^{th}$ century, an introduced fungal pathogen, *Cryphonectria parasitica*, was documented in the New York City Botanical gardens (Merkel 1905). This pathogen was found to be responsible for a novel stem girdling disease in *C. dentata*, termed chestnut blight (Anagnostakis 2012). This disease would go on to spread throughout the native range of *C. dentata* at a rate of 30 km a year, wiping out an estimated 90.0 - 99.9 % of the estimated population of 4 billion American chestnut (Dalgeish et al. 2015; Van Drunen et al. 2017). Within a short 50 yr, this species was reduced from a keystone primary producer and commercially relevant species, to a functionally extinct species existing primarily in the wild as stump sprouts.

The immediate conservation of *C. dentata* requires the efficient clonal propagation of existing select trees with suspected blight resistance (Van Drunen et al. 2017). However, *C. dentata* cannot be clonally propagated efficiently by conventional means. It does not root from cuttings, and has a very low graft success rate (Galic et al. 2014; Galic 2016). An axillary shoot culture system has been developed for this species (Keys and Cech 1982; Serres et al. 1990; Xing

et al. 1997). However, documented microshoots were of low quality and overall multiplication rates were unreported.

The development of individuals with high blight tolerance is a key conservation objective for this species. An efficient protocol for the recovery of plant tissues through somatic embryogenesis would allow for the development of blight tolerant lines through genetic transformation. A somatic embryogenesis protocol has been published for this species (Merkle et al. 1991). A limited number of transformed lines have been generated with increased blight tolerance (Zhang et al. 2013). However, successful production of somatic embryos on explants in published protocols were very low, ranging from 0.02 – 3.00 % (Merkle et al. 1991; Johnson et al. 2007; Merkle et al. 2011). As a result of the limitations on the existing somatic embryogenesis protocol in *C. dentata*, too few genotypes have been successfully used to generate somatic embryos for conservation purposes. As well, there is currently no work detailing the metabolomics of the host-pathogen interaction between *C. parasitica* and *C. dentata*. This lack of knowledge makes it challenging to develop a tolerance strategy and difficult to target an effective virulence or host defence factor in engineering a blight tolerant genotype of *C. dentata*. In this thesis, these issues are addressed in order to improve short term tissue culture conservation methods for the preservation of *C. dentata*.

**1.2 Research objectives**

1. To assess the effect of growth stage (embryo-, seedling-, and mature-stage) of the source material on multiplication rate in axillary shoot culture.
2. To determine the effect of two different incubation temperatures (21 ± 1 °C and 31 ± 1 °C) on multiplication rate across eight different genotypes sampled from the same isotherm of origin.
3. To assess the relative multiplication rate in axillary shoot cultures of 22 genotypes of *C. dentata*.
4. To define a framework for the use of axillary shoot culture for the short term conservation of *C. dentata*.
5. To identify the effect of medium components on the incidence of STN and multiplication rate in axillary shoot culture, including BAP, Ca, Mg, B, and gelling agent (agar, gellan gum).

6. To identify growth regulators (NAA: naphthaleneacetic acid, 2,4-D: 2,4-dichlorophenoxyacetic acid, and TDZ: thidiazuron) and combinations which will result in the production of somatic embryos from microcuttings of axillary shoot cultures.
7. To determine if microcuttings from axillary shoot cultures derived from mature-stage explant tissues can be used to initiate somatic embryogenesis.
8. To identify the metabolomics and mechanisms involved in the host-pathogen interaction between *C. parasitica* and *Castanea* spp.

**1.3 Hypotheses**

1. Growth stage will have a significant effect on multiplication rate; genotypes derived from embryo- and seedling-stage tissues will be relatively more vigorous than genotypes derived from mature-stage tissues.
2. Increasing temperature (to 31 ± 1 °C) will increase multiplication rate in a genotype-independent and isotherm-dependant relationship.
3. Axillary shoot culture multiplication rate is genotype dependant, and as a result more than one axillary shoot protocol will be required to maximize the multiplication rate across the 22 genotypes included in this study. This will provide a framework for the future use of axillary shoot culture in the short term conservation of *C. dentata*.
4. Incidence of STN will be found to be related to both BAP and Ca deficiencies in the culture medium, and additional B or Mg will be found to facilitate the uptake of Ca from the medium.
5. Both concentration and type of gelling agent will be found to have an effect on STN, with agar treatments producing greater multiplication rates and lesser rates of STN.
6. A treatment with high auxin to cytokinin ratio of NAA/BAP will produce somatic embryos from microcuttings of axillary shoot cultures.
7. A low TDZ treatment will induce somatic embryo formation from microcuttings of axillary shoot cultures.
8. An equal combination of 2,4-D/BAP will produce somatic embryos from microcuttings of axillary shoot cultures.
9. It will be demonstrated that somatic embryogenesis can be induced on microcuttings of axillary shoot culture derived from mature-stage tissues.

**10.** Clear virulence factors and host defense factors can be determined from the literature in the host-pathogen interaction between *C. parasitica* and the host *Castanea* genus.

# CHAPTER 2. Literature Review

## 2.1 Tissue culture

### 2.1.1 Overview

Today, tissue culture has diverse applications, and culture requirements for a wide range of cells, tissues, or organs from many species have been determined. Worldwide, tissue culture is responsible for the commercial production of millions of clonal plants (Winkelmann 2006; Cardoso et al. 2018). An increasing number of commercially important plant species are produced efficiently and cheaply through micropropagation, including species of *Acer, Betula, Malus, Pinus, Phalaneopsis, Populus*, *Rhododendron*, *Rosa*, *Rubus*, and *Ulmus*, (Zimmerman 1985; Zimmerman 1997; Cyr and Klimaszewska 2002; Winkelmann 2006; Cardoso et al. 2018). Hundreds of plant species are produced this way, including most plantation and orchard species, many forestry species, and horticultural species comprising sometimes hundreds of cultivars. Some commercial species are only economically viable through tissue culture propagation. For example, many orchid species are very difficult to propagate by seed and slow to propagate by conventional cuttings, but have efficient mass propagation systems that allow them to be sold at affordable prices to consumers (Mohanty et al. 2012). Apart from agronomic or horticultural applications, hundreds of plant species are routinely cultured for research purposes; conservation, germplasm storage, pathogen elimination, genetic transformation, breeding, and basic botanical and microbiological studies (Bajaj 1995; Zimmerman 1997; Winkelmann 2006; De Filippis 2014). Variegation and dwarfism are among the many useful traits introduced to horticultural plant species through tissue culture-based breeding (Michler 1993; Piagnani et al. 2008). Tissue culture is also being used to help propagate and preserve the germplasm of rare plant species such as gum karaya (*Sterculia urens*) and devil's club (*Oplopanax elatus*) through cryopreservation, axillary shoot culture, and somatic embryogenesis (Moon et al. 2006; Polin et al. 2006; Devi et al. 2014). With an increased knowledge of the epigenetic mechanisms involved in tissue culture, researchers are gaining even greater understanding and control of all aspects of tissue growth in tissue culture (Osorio-Montalvo et al. 2018).

Existing tissue culture methodologies can be grouped based on the initial explant and the tissue or organs produced in culture. Two of the most widely used methods in tissue culture include axillary shoot culture and somatic embryogenesis.

### 2.1.2. Axillary shoot culture

Axillary shoot culture proceeds in a similar way to stem cuttings performed in vivo (Hartmann et al. 2002). In both instances, a cutting consisting of one or more nodes is used to produce new shoot tissues through the expansion of lateral buds. However, with stem cuttings, the cutting is able to endogenously produce enough resources to allow for lateral bud expansion, and the initial shoot generally phenotypically resembles the initial cutting. In axillary shoot culture, explants generally require the use of growth regulators in the medium to encourage bud expansion and proliferation new axillary shoots. Furthermore, the axillary shoots undergo changes in epigenetic regulation during culture, resulting in a culture-induced phenotype (CIP) which is phenotypically distinct from the source plant (Donnelly 1990; Donnelly and Tisdall 1993; Wang and Jiao 2015; Osorio-Montalvo et al. 2018). The CIP is a reflection of the in vitro environment during the culturing process. This environment is typically warm, with almost saturated humidity, little or no air movement, reduced gas exchange, and sugars present in the medium. In vitro shoots and plantlets exhibiting the CIP are heterotrophic, with tiny leaves and stems with reduced levels of epicuticular and cuticular wax, a thinner epidermis, stomata with poorly controlled apertures, and loosely organized mesophyll. Some species that are recalcitrant to rooting as traditional stem cuttings can be induced to form roots in culture or following ex vitro transfer, including *C. dentata* (Oakes et al. 2013; Oakes et al. 2016).

Axillary shoot culture involves distinct steps defined by specific tissue development stages and precise growth regulator combinations. These steps were outlined by Murashige in 1974, and further modified by Debergh and Maene (1981). These steps are: preparation of the source plant and explant (Stage 0), culture initiation (Stage I), repeating division and subculture (Stage II), ex vitro preparation (Stage III), and ex vitro establishment (Stage IV).

***Stage 0: Preparation of source plant and explant***

Stage 0 involves the maintenance of the source plant(s) used to initiate culture (Debergh and Maene 1981; Krikorian 1995). For axillary shoot culture, explants consist of one or more dormant buds on a section of stem. Stem cuttings can be taken from juvenile-phase or mature-phase plants. Generally, the younger the growth stage of the tissue used to derive the explant, the greater the chance that the explant will successfully grow in culture (McCown 2000). The most recalcitrant explants are often derived from reproductively mature source plants. For mature-phase source plants, the chance of success can be increased by choosing tissues with a greater expression

of vigorous pseudo-juvenile characteristics, including the current year's growth or water sprouts. Methods such as etiolating have been used to successfully culture previously recalcitrant mature-phase source plants (Ballester et al. 1989; Grzyb et al. 2017). Etiolation is a response in angiosperms to low light conditions. Generally a growth response in newly germinating seedlings under the soil, etiolation can be induced in above ground tissues by shielding tissues from all light for extended periods of growth (Maynard and Bassuk 1987). This results in the growth of pale tissues with lesser chlorophyll concentration, but rapid growth and increased internodal length (Burgess 1985). It is believed that the upregulation of growth factors during etiolation may carry over with tissue explants into culture, resulting in more vigorous cultures than explants from non-etiolated tissues. Changes in relative gene expression is detectable through changes to global DNA methylation. Differences in global DNA methylation have been documented in vivo between source material of different growth stages (Hasbún et al. 2005; Hasbún et al. 2007). It was initially believed that these differences in global DNA methylation carried over into culture, and were responsible for the differences observed in relative vigor. However, recent work with European chestnut (*Castanea sativa*) has demonstrated that after a single round of subculture, global DNA methylation rates between mature (13.7 %) and juvenile-phase tissues (23.0 %) equalize in culture (to 15.0 %), and stay stable through at least 3 subculture rounds (Hasbún et al. 2005). As well as growth stage, the growing location of the source plant has a significant effect on culture success. It is preferred to use source plants maintained in growth chambers or in the greenhouse, to reduce microbial load and associated disinfestation issues.

Tissue culture is conducted under aseptic conditions. As such, potential explants must first be disinfested before they can be successfully introduced into culture. Disinfestation procedures vary widely between species (Schulz et al. 1993). The procedure must be strong enough to kill all surface bacteria and fungi, but tolerable to plant tissues. Disinfestation procedures for woody plant material tend to be more aggressive, with more concentrated reagents and featuring longer treatments periods, than procedures used for herbaceous plant species (Dalal et al. 1992; Xiong et al. 2018). Procedures for woody species generally involve immersion in 70.0 % ethanol for 10 s to 1 min, followed by extended exposure to 10.0 – 20.0 % commercial bleach (active ingredient sodium or calcium hypochlorite) for ≥ 20 min (Schulz et al. 1993). During bleach exposure, the explant may be placed under agitation or vacuum in order to eliminate air bubbles and improve contact with the disinfesting agent. This method is currently the most commonly used in culture,

as it kills a wide range of fungi and bacteria, and is both relatively non-toxic and affordable. In cases where a more extreme surface sterilization methodology is required, such as with plant material with a microbial contaminant that cannot be killed by either ethanol or bleach, there are a range of other options available (Leifert et al. 1994). These are generally only used when the ethanol and bleach methods fail, as many of these compounds are either very toxic, expensive, or both. Some of the more common approaches involve the use of formaldehyde or mercuric chloride, both of which are both phytotoxic and harmful to humans (Caetano-Anollés et al. 1989; Schulz et al. 1993). Generally, any microbial contaminants that survive disinfestation will appear in culture within 2 wk. Endogenous organisms are common and may escape into culture, or surface flora that doesn't ordinarily grow in culture may begin to appear if culture conditions vary; particularly if temperature is increased. Once an explant is successfully disinfested, it is introduced to the Stage I medium to initiate growth and commence the culture.

***Stage I: culture initiation***

Stage I involves initiation of the explant into culture, both establishing rapid growth and demonstrating asepsis (Murashige 1974). Stage 1 culture conditions, unlike Stage 0, are often species specific. Stage I and Stage II culture conditions can be identical, or Stage I may consist of unique factors. Choice of mineral composition in the medium is species-specific, although there are some general mineral compositions (known as basal media) which provide adequate nutrition in culture a wide range of plant species. For example, Lloyd and McCown's Woody Plant (WPM) Basal Medium has been found to support growth in a large number of woody plant species. (Lloyd and McCown 1980; McCown 2000). Stage I medium may contain a greater concentration of sugar (generally sucrose) than Stage II. For woody plant species entering Stage I axillary culture, cytokinins are often essential for bud break and shoot elongation (Krikorian 1995). BAP is the most common cytokinin used in culture, although zeatin (Z), kinetin (K), TDZ, or N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU) are sometimes used (Yang et al. 2009). Concentrations of cytokinins required are generally very low; at nano- or micro-molar levels. High concentrations of cytokinins are toxic to plant tissues and result in necrosis. Some plant species require both cytokinin and auxin in Stage I medium (Fujiwara and Kozai 1995). In these cases, cytokinins are usually present at concentrations that are several fold greater than auxin. Auxins utilized include 2,4-D, 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), and NAA (Yang et al. 2009). In rare cases, low levels of gibberellic acid ($GA_3$) are added for shoot extension growth (Krikorian 1995). Some

species do not require growth regulators in Stage I medium. Presumably, these plants synthesize all the hormones required to initiate growth. Plant growth varies depending on the species, genotype, and developmental stage of the source plant. Duration of Stage I is generally 4 – 6 wk. At this time, the explants will have initiated growth and buds may have elongated into a small shoot. The shoot is then subcultured; cut into sections and transferred into Stage II medium.

***Stage II: repeating division and subculture***

In Stage II of axillary shoot culture, repeated division and subculture of microcuttings occurs (Murashige 1974). The goal of Stage II is to produce fully expanded axillary shoots, either for repeated subculture or for transfer ex vitro. The axillary shoot produced in Stage I is divided into one to several node stem cuttings. These stem cuttings become the initial microcutting for Stage II. From these microcuttings, new axillary shoots are produced. Once Stage II is underway, subculture is repeated every 4 – 6 wk. Stage II can be repeated indefinitely, with a target increase rate of ≥ 10 X (considered a commercial increase rate). For other species the multiplication rate and culture vigor may decline over successive subcultures (Huang et al. 1992; Thomas 2004). In some species, fresh explants are used to initiate Stage I at regular intervals. In other species, Stage II cultures are introduced to a medium high in cytokinin for a single subculture cycle, providing a so-called "cytokinin pulse" to stimulate growth (Zhang and Finer 2016). Microcuttings that produce axillary shoots large enough to be subcultured for another round of Stage II, are either subcultured to Stage II again, transferred into Stage III to prepare for transfer to Stage IV, or transferred directly to Stage IV ex vitro.

***Stage III: ex vitro preparation***

Stage III in axillary shoot culture is an intermediate step between Stage II and Stage IV, and is used to prepare Stage II shoots for survival ex vitro in Stage IV (Murashige 1974). Protocols for Stage III generally involve inducing rooting and preliminary hardening off the rooted plantlet for transfer to the greenhouse (Krikorian 1995; Smith 2013). Stage III medium is similar to Stage II medium with important modifications. Cytokinins necessary for Stage II may be eliminated completely or their levels decreased, and additional auxin added to stimulate rooting. Methods must sometimes be used to remove excess cytokinin from the explant before rooting will take place. Plantlets may be grown on medium containing activated charcoal to absorb excess growth regulators, before rooting treatments (Thomas 2008). Specific abiotic (light) and chemical treatments have also been used to promote rooting success and adjustment to the greenhouse. It is

common to eliminate light to the medium (using charcoal) or to the entire culture (placing containers in the dark), while reducing relative humidity (RH) in the culture environment (Xing et al. 1997). Paclobutrazol (PBZ) is a plant growth retardant, that is also used as a chemical additive in Stage III to increase rooting rate in plantlets. For example, Philodendron treated with PBZ in vitro had a greater rooting and survival rate (100.0 %) compared with controls (78.0 %) (Ziv 1992). Plantlets treated with PBZ are generally shorter and have thicker stems than their control counterparts. However, they also have greater root density, resist wilting, and have greater survival ex vitro during Stage IV (Ziv 1992; Eliasson et al. 1994; Kozak 2006; Marín et al. 2016). Growth may continue to be negatively affected by PBZ treatment even after successful acclimatization, which has limited its use as a rooting aid (Ziv 1992; Eliasson et al. 1994).

During the early to mid-20$^{th}$ century, Stage III was necessary for many plant species. Most early procedures had yet to determine the optimal methods for rooting or transferring plants ex vitro, requiring additional steps to optimize survival ex vitro. Modern axillary shoot procedures are generally optimized to create Stage II shoots sturdy enough to be moved directly to Stage IV, excluding Stage III entirely (Smith 2013).

***Stage IV: ex vitro establishment***

Stage IV in axillary shoot culture involves the transfer of shoots or rooted plantlets to the greenhouse (ex vitro) from Stage II or Stage III (Hartmann et al. 2002). During Stage IV, transplanted plantlets gradually outgrow the CIP and trend towards a new phenotype that reflects the transplant environment (Pospóšilová et al. 1999; Pospóšilová et al. 2007). For many species this is the most difficult step in micropropagation, with significant losses even in relatively successful procedures (Pospóšilová et al. 1999; Oakes et al. 2016). Stage IV is highly species-specific, although many protocols share similarities. Survival generally relies on careful and gradual acclimatization to lower RH, higher light levels, and roots must be produced on microcuttings lacking these in culture (Gonçalves et al. 2017).

Most transplants must be gradually exposed to decreasing RH levels until new leaves with greater cuticular and epicuticular waxes, increased stomatal function, and increased mesophyll packing density can develop (Gonçalves et al. 2017). However, chemical treatments can also be used to induce acclimation. In chrysanthemum plantlets, the addition of PBZ was shown to improve stomatal function versus control plants (Kucharska and Orlikowska 2008). Biotic symbionts have also been used to increase Stage IV growth rate and survival (Krishna et al. 2005).

In common grape (*Vitis vinifera*) axillary shoot culture, multiple-species mixtures of arbuscular mycorrhizae significantly increased several growth markers in shoots during Stage IV transfer. Specifically, mycorrhizae treated shoots accumulated greater concentrations of nitrogen, potassium, magnesium, and iron, and had greater content of chlorophyll, carotenoids, and an improved overall photosynthetic rate. Some plants are highly capable of conserving water even immediately out of culture and therefore do not need stringent acclimatization to ambient humidity (Krishna et al. 2005).

In addition to lower ambient humidity, in many protocols ex vitro transplants also need to be acclimatized to greater light levels (Gonçalves et al. 2017). When grown in vitro, shoots experience relatively low light levels and this often results in CIPs that include thinner leaves with less chlorophyll content, leaf mesophyll with reduced packing density and lower photosynthetic rate (Singsit and Veilleux 1991; Tichá and Kutík 1992; Donnelly and Tisdall 1993; Noé and Bonini 1996; Pospóšilová et al. 1999). As with lowered humidity, plantlets are typically acclimated to greater light levels through a gradual increase. Morphological adaptations to greater light levels are less well studied than water retention. Nevertheless, considerable changes have been documented to occur during this process. For most plant species, acclimatization to increased light levels includes changes in internal morphology to maximize chlorophyll and other pigment content, and photosynthetic rate (Pospóšilová et al. 1999; Guan et al. 2008; Gonçalves et al. 2017). For example, American sweetgum (*Liquidambar styraciflua*) plantlets in culture had thin leaves, the mesophyll cells had large vacuoles, minimal cytoplasm, and flattened chloroplasts with abnormal internal membranes (Wetzstein and Sommer 1982). Once the transplants had acclimated, new leaves formed ex vitro were thicker, the mesophyll tissue differentiated into palisade and spongy parenchyma cells with appropriate vacuoles and chloroplasts with true grana. However, there are some exceptions to this. For example, peace lily (*Spathiphyllum floribundum*) plantlets were as photosynthetically competent in vitro as were plants ex vitro, and normal source-sink relations were observed in in vitro plantlets (Van Huylenbroeck et al. 1998). If light is increased too quickly or significantly, transplants can experience photoinhibition (Gonçalves et al. 2017). Photoinhibition leads to an increase in reactive oxygen species (ROS) within shoot tissues, which can result in a range of issues including DNA and protein damage, enzyme inactivation, and membrane injury (Batková et al. 2008).

Finally, for species which are not rooted in vitro, roots must grow ex vitro (Oakes et al. 2016). When rooting occurs during acclimatization, the rate of acclimatization may be slowed (Pospóšilová et al. 1999). For example, during the acclimatization of *S. floribundum* plantlets, two different growth stages were observed (Van Huylenbroeck and De Riek 1995). During the root formation stage, leaf growth occurred slowly. Once roots had developed to a certain degree, both shoot and root formation quickened. During ex vitro rooting, unrooted transplants are treated as conventional stem cuttings. Rooting hormone powders containing indole-3-butyric acid (IBA) are used to help induce rooting, and transplants are protected from water loss through high ambient humidity (although still less than the humidity during culture; Oakes et al. 2016). In species which form callus balls as opposed to roots in vitro, the ball may or may not be removed to stimulate rooting (Oakes et al. 2013; Oakes et al; 2016). In some species, root formation seems to occur primarily on the stem above the callus ball and in fact the presence of the ball may inhibit root formation or may be neutral to root formation (callus is facultative to rooting). However, for other species, roots emerge directly from the callus ball and its presence is needed for root formation (callus is obligatory to rooting).

Once a transplanted microcutting has rooted successfully and acclimated to transplant conditions, the final process in outgrowing the CIP involves the gradual enlarging of plant organs and continued phenotypic change towards growing conditions typical of the transplant environment. For most species, this is achieved through continuous production of new organs. Each successive leaf flush increases in size and stem and root diameter widens accordingly. During and after Stage IV, transplants trend towards the original source plant, both phenotypically and biochemically (Gonçalves et al. 2017).

### 2.1.3 Somatic embryogenesis

Somatic embryogenesis can be defined as a process where, without a fusion of gametes, haploid or diploid somatic cells can develop into polar structures resembling zygotic embryos, and differentiate into full plants through a process resembling the stages of zygotic embryo development (Smertenko and Bozhkov 2014). This is a process which occurs naturally as part of the life cycle in many plant species, or can be induced through stress conditioning (Garcês and Sinha 2009; Fehér 2015). Comparative DNA studies have noted that although somatic embryogenesis may phenotypically resemble zygotic embryogenesis, there are differences in gene expression between the two processes (Cairney et al. 1999). A study comparing DNA expression

during somatic and zygotic embryo induction, formation, and maturation in loblolly pine (*Pinus taeda*) noted that somatic embryogenesis involved a difference in gene expression in 10.0 % of the 6000 bands examined. In tissue culture, somatic embryogenesis can be induced in plant species which otherwise seem incapable of somatic embryogenesis under field conditions. In tissue culture, somatic embryos are used primarily for breeding and propagation, where germplasm fidelity is not essential (Corredoira et al. 2017).

Somatic embryogenesis is utilized in several breeding strategies. Protocols for genetic transformation systems frequently utilize somatic embryogenesis to regenerate transformed cells into complete plantlets (Polin et al. 2006). Somatic embryogenesis can also result in the production of mutants in regenerated plants, resulting in what are termed somaclonal variants (Hashmi et al. 1997; Fehér 2015; Corredoira et al. 2017). The generation of somaclonal variants is an important breeding tool for self-incompatible plants. It has also been used for plants which can only propagate clonally, such as banana (Del Carmen Vidal and García 2000).

The development of somatic embryos from somatic tissues is a complex process. An explant consisting of somatic cells is induced in culture to form a bipolar structure resembling a zygotic embryo with a shoot and root apex, but without a vascular connection to the original tissue (Smertenko and Bozhkov 2014). The embryo can form directly on the explant (direct embryogenesis) or on callus which develops on the explant (indirect embryogenesis) (Smertenko and Bozhkov 2014; Shajahan et al. 2016). This structure then undergoes growth through the stages of embryo development (globular, heart, torpedo, cotyledonary), before regeneration into a plantlet (von Arnold et al. 2002; Fehér 2015). However, cotyledons are often reduced in size, and both endosperm and seed coat fail to develop (Carville 1975; Hartmann et al. 2002).

Similar to axillary shoot culture, the procedure of somatic embryogenesis can be divided into distinct Stages defined by the particular stage of tissue growth occurring in culture (Smertenko and Bozhkov 2014; Shajahan et al. 2016). Stages generally require different media, and even different abiotic culture conditions (Murashige 1974; Debergh and Maene 198; Smertenko and Bozhkov 2014; Shajahan et al. 2016). Stage 0 is common to both somatic embryogenesis and axillary shoot culture, and is not detailed below. However, Stages I, II, and III can differ significantly between axillary shoot culture and somatic embryogenesis. The specifics of these Stages for somatic embryogenesis are outlined below.

***Stage I: culture initiation and initial callusing***

Explants utilized for somatic embryogenesis can vary widely, particularly in the case of indirect somatic embryogenesis (Smertenko and Bozhkov 2014). Single somatic cells, haploid gametes, sections of immature zygotic embryos such as the cotyledons, ovary tissues, and differentiated mature tissues have all been used successfully as explants in somatic embryogenesis. There is some confusion in the application of the term somatic embryo in the literature. Embryo structures arising directly from the embryo section of immature zygotic embryos explants have been defined as either adventitious zygotic embryos or somatic embryos (Sharp et al. 1980; Hartmann et al. 2002). This review will be detailing somatic embryos derived from explants of true somatic tissues. However, it is important to note that not all studies specify the explant source of their adventitious embryos, and in fact they may be working with adventitious zygotic embryos as opposed to true somatic embryos.

Stage I in somatic embryogenesis involves the proof of disinfestation and explant establishment also seen in axillary shoot culture (Murashige 1974). However, in somatic embryogenesis, successful culture induction is defined as the production of callus (indirect embryogenesis) or the first stages of embryo emergence (direct embryogenesis) (Montalbán et al. 2016). Biologically, callusing is a natural response to wounding stress (Fehér et al. 2003). Factors such as wounding, osmotic stress, growth regulators, and even organic components in the medium all contribute to explant stress. Stress in turn reprograms gene expression, which has been shown to affect cell physiology, metabolism, source/sink regulation, and ultimately cell division and development. Different regulators have been identified for somatic embryogenesis at the transcription level (Smertenko and Bozhkov 2014). Regulators can vary by species. However, they can also vary within the same species depending on the specific protocol utilized for somatic embryogenesis (for example, direct vs indirect protocols). For example, with the model organism thale cress (*Arabidopsis thaliana*), EMBRYOMAKER of the AP2/ERF family of transcription factors, is associated with both direct and indirect embryogenesis, while LEAFY COTYLEDON1 of the HAP3 domain transcription factor is associated only with the induction of direct somatic embryogenesis, and short term expression of RKD4 of the RWP-RK domain transcription factor promotes somatic embryogenesis in the absence of auxin (Lotan et al. 1998; Tsuwamoto et al. 2010; Waki et al. 2011). Induction media generally require high auxin levels, although in some instances somatic embryos can be generated in the complete absence of auxin (Dodds and Roberts

1995; Waki et al. 2011). Cytokinins can be used in some cases to increase the rate of cell division, but cell response to cytokinin is very species-specific and generally cytokinin concentration must be lower than auxin concentration (Sharp et al. 1980; Montalbán et al. 2013). Other growth regulators (such as abscisic acid: ABA) are sometimes beneficial or necessary, but the response is also species-specific (Bhojwani and Razdan 1996; Fehér et al. 2003). Indirect embryogenesis generally required higher concentrations of growth regulators compared to direct embryogenesis (Sharp et al. 1980). Differences in growth regulator concentrations, even within the same species, is likely a function of the inherent mechanistic differences in direct and indirect embryogenesis (Smertenko and Bozhkov 2014). The initiation of direct embryogenesis generally involves adding an inducer or removing a suppressor to embryogenic potential, while indirect embryogenesis is a two-step process that requires callus growth and then embryo induction. The optimal concentrations of growth regulators are generally species-specific, and is often only determined by trial and error (Montalbán et al. 2013). Excessively high levels of growth regulators can inhibit growth, while suboptimal levels will result in disorganized callus formation. Within the induction medium, auxin initiates the process of embryo formation, but also inhibits embryo development past the globular stage (Sharp et al. 1980; Dodds and Roberts 1995). At this point, cultures for many species must be moved to a different medium (Stage II) in order to continue development or maintain the embryogenic potential of the established cultures, although in some species embryogenic potential can be maintained on the same Stage I medium (Nunes et al. 2018).

***Stage II: embryo formation and growth***

Callus produced in Stage I must generally be moved to a different Stage II medium for embryo formation or embryo development beyond the globular stage (Sharp et al. 1980). Although, for some species, Stage I and Stage II media are the same (Montalbán et al. 2013).

Stage II medium is highly species and explant specific. The growth regulators necessary for some species to develop embryos may even be detrimental to other species. Even within the same species, different explant sources (particularly between indirect and direct embryogenesis) may require completely different Stage II media (Smertenko and Bozhkov 2014). Even within a single class of compounds, different particular compounds may elicit completely different responses within the same species (Montalbán et al. 2013). For instance, the cytokinin Z stimulates embryo production in carrot cultures while two different cytokinins, K and BAP, inhibit embryo production (Kato and Takeuchi 1963; Sharp et al. 1980). However, generally Stage II medium

involves lower or absent auxin concentrations relative to the Stage I medium (Dodds and Roberts 1995). The formation of embryos is associated with the development of cellular polarity, and the polar transport of auxin (de Jong et al. 1993). The correct polar transport of auxin leads to asymmetrical cell division and encourages embryo development beyond the globular stage (Schiavone and Cooke 1987; Michalczuk et al. 1992; Liu et al. 1993). Once callus or proembryogenic cells have developed embryos at either torpedo-stage or cotyledonary-stage, the somatic embryos are then transferred to an embryo germination medium.

***Stage III: embryo germination***

Generally, embryos are not capable of growth beyond the embryo stage (germination) in the Stage II medium. Stage III is utilized to refer to the tissue stage where somatic embryos from Stage II are germinated into a full plantlet with roots and shoots.

Efforts may be necessary to synchronize the development of embryos at this stage, in order to facilitate Stage IV by utilizing tissues of similar physiological developmental stages (Alwael et al. 2017). Synchronization involves the establishment of cultures of embryos which are developmentally similar (for example, all in the torpedo stage). Some species synchronize without external stimulation, and some are recalcitrant to synchronization and require external factors to aid synchronization (Colova et al. 2007; Klimaszewska et al. 2016). Synchronization factors are highly species specific. Stage III medium formulation is often identical to the medium used for micropropagation of shoots of the species in question, as cotyledonary-stage somatic embryos are fully formed and only require the removal of the inhibitory effects of other embryos or the embryo initiation medium in order to germinate (Nassar et al. 2011). However, some species require additional treatments in order to germinate (Xing et al. 1997).

The factors which induce embryo germination vary widely between different species and do not necessarily correspond with embryo germination in the source plant. For instance, ABA inhibits germination in zygotic embryos (Hartmann et al. 2002). However, ABA has been added to some Stage III media to improve the quality of plantlets regenerated from somatic embryos (Ammirato 1974; Ammirato 1983; Robichaud et al. 2004; Liao and Juan 2014; Carneros et al. 2017). By delaying germination until embryos are fully developed, ABA improves the quality of somatic embryos formed and can even be used to synchronize embryo germination (Ammirato 1974; Ammirato 1983; Alwael et al. 2017). Auxin inhibitor, 2-(4-chlorophenoxy)2-methylpropionic acid (PCIB), has been utilized during Stage III for similar reasons (Carneros et

al. 2017). Treatments independent of the media can also be used to overcome somatic embryo dormancy. Abiotic factors such as temperature shock, desiccation, and osmotic stress overcome dormancy in some species (Liao and Juan 2014; Carneros et al. 2017). For example, Liao and Juan (2014) found that somatic embryo germination in Taiwan spruce (*Picea morrisonicola*) increased from < 10.0 % to 69.9 % with a combination of cold treatment at 4 °C for 3 wk followed by 1 wk in a vented petri dish for partial embryo desiccation. Overall embryo germination rates can be very low and generally vary between species and even among genotypes. Once germinated, microshoots or plantlets are generally maintained through axillary shoot culture. Methods for the Stage IV transfer of microshoots or plantlets derived from somatic embryos are similar to methods derived for axillary shoot cultures.

### 2.1.4. Shoot Tip Necrosis

Physiological problems, among them hyperhydricity (vitrification) and STN, can develop in shoots or plantlets during culture (Gaspar et al. 2002; Kevers et al. 2004; Jan et al. 2018). STN is particularly problematic with woody plant species and involves localized necrosis of the apical shoot tip (McCown and Sellmer 1987; Bairu et al. 2009b). The shoot or plantlet may survive and continue to grow after the tip has died back. In some cases, new shoots may develop and considerable branching may occur just below the former apex (McCown and Sellmer 1987; Vieitez et al. 1989; Jan et al. 2018). These secondary shoots can then go on to surpass the length of the original apex.

Susceptibility to STN varies between species, genotypes, and even individual clones (Reed et al. 2013). Generally, the larger or more vigorous a culture, the greater the prevalence of STN (McCown and Sellmer 1987; Jan et al. 2018). Although a plantlet with STN may continue to grow, the compromised quality generally reduces the in vitro multiplication rate, and produces smaller microshoots which are often more difficult to root or transfer ex vitro (Biondi et al. 1984; Chevré 1985). Necrosed tips are also more vulnerable to fungal infection, which can spread to adjacent transplants during ex vitro treatments (Chevré 1985). Although the pruning of individual branch tips before ex vitro transfer is possible, this is not economically feasible in commercial systems (McCown and Sellmer 1987). For species susceptible to STN in culture, major efforts are made to reduce or eliminate this physiological problem.

STN in axillary shoot cultures is often found to be a complication of Ca deficiency (McCown and Sellmer 1987; Bairu et al. 2009b). Ca is a relatively immobile micronutrient, and

extreme deficiency symptoms may involve shoot tip death (Leifert et al. 1995). In the field or greenhouse, Ca is moved efficiently in the plant through transpiration. The high RH in culture severely limits transpiration in vitro (McCown and Sellmer 1987). As a result, Ca is not effectively transported to the actively growing shoot tips. This situation is aggravated when growth is very rapid. Several solutions to the problem of STN have been proposed, all of which potentially improve Ca transport to developing shoot tips. One strategy involves modifying the culture environment to reduce RH, resulting in the promotion of shoot transpiration rates. Examples of this strategy include actively or passively venting the lids of culture vessels. Microcuttings of *Dianthus caryophyllus* x *barbatus* grown in containers with a vented lid had greater levels of cellular Ca and lower rates of STN than in un-vented control cultures (Cassells and Walsh 1994). However, STN can also be caused by excessive venting (Mills et al. 2001). Jojoba (*Simmondsia chinensis*) plantlets developed STN localized near container lids that were vented. Venting is also a method which has been employed to minimize aspects of the CIP (Donnelly and Tisdall 1993; Majada et al. 2000). For instance, in vented culture vessels stomatal function and cuticle thickness was increased, which increased transplant success rates during Stage IV (Cassells and Walsh 1994; Majada et al. 2000). In potato (*Solanum tuberosum*) plantlets, hyperhydricity symptoms were completely eliminated through the use of vented lids (Park et al. 2004). Venting can reduce the incidence of STN in some species, and also mitigate some of the humidity-related negative physiological conditions related to the CIP. However, venting systems can be costlier than traditional stationary container systems, and are not effective with all species. On the whole, they have not been widely implemented. Another strategy is to slow the growth of cultures to minimize the incidence of STN (McCown and Sellmer 1987). This is a counterproductive method that supresses the micropropagation rate and is perhaps only useful for extremely valuable specimens, where survival is more important than overall multiplication rate.

Other methods involve modifying medium components to make Ca more available for uptake (McCown and Sellmer 1987; Kovalchuk et al. 2017; Jan et al. 2018). These methods can be as simple as diluting the medium to make Ca more available, or adding more Ca to the medium. Kovalchuk et al. (2017) found that adding $CaCl_2$ at a rate of 2.7 X the basal amount in their WPM (to 259.7 mg/L from 96.0 mg/L) eliminated the incidence of STN in wild apricot (*Prunus armeniaca*) axillary shoot cultures, while also increasing leaf size. However, excessive levels of Ca can also increase the incidence of STN (Bairu et al. 2009a). As well, different Ca salts can also

have different effects on STN in culture (Barghchi and Alderson 1996). With pistachio (*Pistacia vera*) microshoots, $Ca(C_2H_3O_2)_2$ and $CaCl_2$ in the medium were absorbed differently by the plant. Both Ca treatments reduced STN. However, $Ca(C_2H_3O_2)_2$ reduced shoot growth overall relative to the $CaCl_2$ treatment. In other species, $CaCl_2$ as a source of Ca has caused chlorine (Cl) toxicity (Chiruvella et al. 2011). Cl is a micronutrient required in small concentrations for plant growth. Cl toxicity can exacerbate the incidence of STN, as well as resulting in brittle stems and yellowing plantlets.

The role of boron (B), which is co-transported with Ca, has been actively investigated for its role in STN (Barghchi and Alderson 1996; Abdulnour et al. 2000; Bairu et al. 2009b). With *P. vera*, both added Ca (as $Ca(C_2H_3O_2)_2$) or added B (as borate) reduced the incidence of STN (Barghchi and Alderson 1996). Doubling Ca from 3.0 mM to 6.0 mM significantly reduced levels of STN but did not affect shoot growth. However, while additional B (700.0 µM) reduced the incidence of STN (from 2.6 % to 0.4 %), it also reduced shoot proliferation (from 11.4 to 6.4 shoots per microcutting). While additional Ca or B separately added to the medium reduced the incidence of STN in devil's claw (*Harpagophytum procumbens*) plantlets, when added together they had a negative effect on shoot growth (5.3 cm average shoot length in the negative control vs 3.6 cm for 15.0 mM Ca and 0.5 mM B) (Bairu et al. 2009a). In a culture system for *S. tuberosum*, it was noted that additional B (0.025 mM to 0.3 mM) had a negative effect on Ca uptake unless the B-free gelling agent Gelrite was used instead of agar (Abdulnour et al. 2000). While the exact relationship between Ca and B in the plant remains to be understood, there seems to be a clear relationship between these two compounds in culture (Bairu et al. 2009a). At a low level of B, Ca uptake by the plant is facilitated. However, too much B limits foliar Ca uptake and drastically limits shoot growth in a species-specific manner. The line between plant toxicity and deficiency for B is the narrowest of all the micronutrients, which may explain some of the difficulties in adjusting B levels to reduce STN (Hartmann et al. 2002). B requirements in some plant species may be so minimal, that the natural endogenous levels of B in some gelling agents may already be at a level detrimental to growth (Abdulnour et al. 2000). Many common basal medium recipes include B, which combined with gelling agents that also contain trace amounts of B exacerbate STN issues for susceptible species (Leifert et al. 1995). When STN is problematic, media formulations need to be examined in detail and B and Ca levels as well as gelling agents carefully adjusted.

In addition to directly modifying the mineral concentrations in a medium, the concentrations and types of growth regulators (auxins, cytokinins, and gibberellins) can also be adjusted, to minimize STN while also allowing for satisfactory levels of shoot growth (Barghchi and Alderson 1996; Bairu et al. 2009b). Foliar applications of cytokinin have reduced rates of STN, but may interfere with subsequent rooting performance during Stage IV (Bangerth 1979). In mature apple trees, a foliar spray of BAP or K increased foliar Ca levels (Shear and Faust 1970; Bangerth 1979). Vieitez et al. (1989) noted that added 0.01 – 0.03 mg/L of BAP to the medium during axillary shoot culture reduced rates of STN in *C. sativa* and English oak (*Quercus robur*). However, treated shoots had reduced rooting rates compared with untreated controls. For *C. sativa* entering Stage IV, a quick dip for apical shoots tips in 0.03 mg/L of BAP completely eliminated the incidence of STN, but reduced rooting from 97.0 % to 25.0 %. Piagnani et al. (1996) reduced the % STN in *C. sativa* plantlets during ex vitro rooting by locally applying 5 µmol of BAP directly to shoot tips. In one experiment, STN was completely eliminated compared to a STN incidence rate of 76.7 % in the control. Although the foliar application of BAP is an effective method to reduce or eliminate STN in culture, its negative effects on shoot growth and rooting rate in some species has prevented the widespread application of foliar BAP as a treatment for STN.

The utility of tissue culture for agriculture and horticulture is well established. However, tissue culture has only recently been applied for the conservation of plant species (Corredoira et al. 2017). Taking into consideration future losses predicted in plant species diversity as a result of climate change and human-mediated disturbances, conservation is likely to become an important use of tissue culture in the future.

**2.2. Tissue culture and conservation**

Estimations of plant extinction rates in the coming century range broadly and have changed significantly over time (Lucas and Synge 1978; Brummitt et al. 2015). The most recent research suggests that 20.0 % of all known plant species are currently threatened with extinction (Brummitt et al. 2015). Particular characters unique to plants, such as the long term storage of germplasm in desiccation-tolerant seeds, allows for the use of conservation strategies not available in other organisms (Dekkers et al. 2015; Magnani 2018).

Many different conservation programs have been proposed based on ex situ seed storage (Li and Pritchard 2009). The largest of these programs is the GSPC, created in 2002 as part of the United Nation's Convention on Biological Diversity multilateral treaty. This project has many

distinct goals involving the conservation of all classes of plant species. With plants currently classified as threatened, it is a goal of GSPC to have seed samples of 75.0 % of all threatened plant species collected and placed into storage. However, seed banking is not an option for many species (Wyse et al. 2018). Most tropical species have desiccation-sensitive (recalcitrant) seeds which cannot survive the drying process necessary before cold storage. Some species produce seeds that are not adapted to survive dormancy for extended periods of time (Li and Pritchard 2009; Dekkers et al. 2015). Alpine species, a group significantly at risk from climate change in the 20$^{th}$ century, generally have shorter seed viability periods than their lowland counterparts (Mondoni et al. 2011). Some valuable species, such as the triploid banana species, do not produce seeds at all and are propagated vegetatively (Engelmann 2004). Finally, some species cannot be selfed and thus produce only heterozygous seed, which may be undesirable if the conservation of a specific genotype is desired, for instance an individual of *U. americana* suspected of being tolerant to Dutch elm disease (Engelmann 2004; Whittemore and Olsen 2011). For these species which cannot be conserved through seed storage, tissue culture provides a possible mechanism for germplasm conservation and breeding (Corredoira et al. 2017; Funnekotter et al. 2017; Kushnarenko et al. 2018).

No single strategy has been proposed for the use of tissue culture in conservation. Instead, several approaches have been suggested based on desired end goals and available resources. These approaches can be divided into short term conservation, medium term preservation, and long term preservation (Rajasekharan and Sahijram 2015; Corredoira et al. 2017).

### 2.2.1. Short term conservation

Short term conservation involves techniques which emphasize rapid propagation and breeding utility over more stable, long term storage options. Both axillary shoot culture and somatic embryogenesis are frequently used for short term conservation. Axillary shoot culture is optimal for germplasm stability over time (Corredoira et al. 2017; Deb et al. 2018). It is currently documented as the most genetically stable form of tissue culture. Axillary shoot culture can also be seen as superior to seed conservation, even in species where seeds can be easily conserved. Through seed propagation, a single embryo can only produce a single individual. However, utilizing tissue culture, a single seed can act as an explant for the creation of many clonal copies of that individual, which can then subsequently be replanted into the field. *Rhododendron wattii* is an endemic species in India (Mao et al. 2018). Only 4 individuals were known to exist in 2012.

Population loss was attributed to overharvesting rather than abiotic factors such as climate change (Mao and Gogoi 2007). As such, reintroduction was considered to be a viable option. Seeds were used as explant material to start axillary shoot cultures of this species in 2014 (Mao et al. 2018). Plantlets were screened for deleterious mutations or abnormal phenotypes utilizing inter simple sequence repeat (ISSR) fingerprinting. No difference was found between the seedling source material and the cultured plantlets growing ex vitro. 60 new plantlets were generated from these cultures, and were reintroduced to the area surrounding the remaining trees in May 2016. Plants were observed to establish and continue growth through to the end of 2016, when the study ended. In a 2 yr span, the remaining population of a rare endemic plant species was increased by 15 fold. This species readily self-pollinates, so these 60 plantlets truly represent 60 new reproductively-capable individuals in the existing population. Establishing a species in axillary shoot culture can also create a tissue repository of known culture-responsive phenotypes for further manipulation in culture (McCown 2000). Another of the critical uses of axillary shoot culture in conservation is to provide a reliable source of material to initiate somatic embryogenesis.

Somatic embryogenesis in conservation is typically utilized for very different purposes than axillary shoot culture (Corredoira et al. 2017). Mutation rates during somatic embryogenesis are often considerable. This makes the technique not optimal for germplasm conservation, as novel mutations can be detrimental and lower the quality of the resulting plantlets. However, the generation of genetic variation can also be a useful breeding and conservation tool (Ali and Ray 2018; Żabicki et al. 2018). Somatic embryogenesis has been proposed as a directed breeding tool to help wild species adapt to climate change by improving generating mutations with increased tolerance to temperature or soil moisture extremes (Ali and Ray 2018). Even if only neutral mutations are generated through somatic embryogenesis, these mutations represent an increase in genetic diversity. Żabicki et al. (2018) in their study on the rare European native, *Viola stagnina*, noted that all plantlets regenerated from somatic embryos displayed some degree of genetic difference from their source plants, although they did not differ phenotypically (even after 2 generations). As well, the range of genetic variation in these regenerated somaclones was statistically similar to the range of genetic variation found in the source population. Żabicki et al. (2018) suggest that through somatic embryogenesis, they have reproduced (although much more rapidly) the degree of genetic variation naturally produced through sexual reproduction. They further suggest that such a program could be used to increase natural genetic variation in a species,

assuming that deleterious mutations were carefully rogued during an initial assessment phase under greenhouse conditions (Mao et al. 2018). For species which are under threat from an introduced pathogen, the insertion of one or more resistance gene(s) could help conserve the species (Merkle et al. 2007). For these species, somatic embryogenesis is a valuable tool for recovering transformed tissues and conversion to plantlets.

### 2.2.2. Medium term preservation

The purpose of medium term preservation is to significantly reduce the growth rate of a culture, so that it can be stored over a significant period of time without the need for subculture (Corredoira et al. 2017). The length of time between subcultures can vary considerably between species. However, medium term preservation programs ideally strive for storage periods on the order of months to years. These procedures are designed to limit the costly investment of time and resources required for the storage of germplasm as active cultures.

Medium term preservation is typically accomplished by modifying abiotic factors in the environment to reduce growth (Corredoira et al. 2017). These can include reducing light levels (even to full darkness), reducing temperature, applying osmotic stress, and modifying the partial pressure of oxygen in the atmosphere. For example, axillary shoot cultures of *C. sativa* were stored at 8 °C for 48 mo, and recovered at a success rate of 82.0 % (Capuana and Di Lonardo 2013). Cultures stored at 4 °C did not survive past 12 mo. Often multiple components can be modified in a single system. Black alder (*Alnus glutinosa*) axillary shoots were recovered at a rate of 75.0 – 87.0 % after 18 mo when stored at 4 °C under low light (8 – 10 µmol $m^{-2}s^{-1}$) relative to axillary shoot culture standards of 25/20 °C day/night temperatures and light levels of 60 – 80 µmol $m^{-2}s^{-1}$ (San José et al. 2015). In some cultures, plant material can also be modified directly to slow growth, such as through partial desiccation. Medium components have also been modified. This generally involves a reduction of mineral or carbon medium components, the addition of growth retardants, changes to growth regulators, or the addition of osmotically active compounds. Musli (*Chlorophytum borivilianum*) microshoots were preserved for 4 mo by elevating the sucrose concentration of the medium to 120.0 g/L (from 30.0 g/L; Chauhan et al. 2016).

### 2.2.3. Long term preservation

In long term preservation, the extended storage of material for later revival is emphasized (Reed et al. 2008). This method relies on techniques to arrest cell division and metabolic processes without killing the tissues in question. Generally this involves cryopreservation; storage of tissues

at a very cold temperature (typically -196 °C) (Engelmann 2004; Funnekotter et al. 2017). Cryopreservation involves limited to no human input after initial set-up, requires very little space, and can theoretically preserve tissues indefinitely, making it a powerful tool for conservation (Merkle et al. 2017).

In cryopreservation, tissues must go through various stages of preparation before they can be safely frozen for eventual recovery (Engelmann 2004; Funnekotter et al. 2017). These stages are often species specific. However, they are mainly employed to achieve one goal, the dehydration of plant tissues. Water expands when frozen. It is this mechanism which typically prevents the successful freezing and thawing of living biological material. As intracellular water freezes, it expands in the form of ice crystals, and these crystals can lyse cells (Storey and Storey 1992). Therefore when a previously frozen organism thaws, tissue damage left from freezing is generally extensive, resulting in the death of the organism. Animals which survive freezing as a part of their natural life cycle typically have cellular mechanisms such as the supercooling of bodily fluids, minimizing ice crystal formation, the partial desiccation of extracellular spaces, or have enzymes which produce ice crystals with rounded edges. Most plant seeds, excluding the recalcitrant seeds, have desiccation mechanisms which result in very little water remaining in seeds during dormancy (Engelmann 2004). As a result, seeds can be stored below freezing with a relatively simple drying pre-treatment (Michalak et al. 2015). However, for plant species where seeds are unsuited or unavailable for cryopreservation, vegetative plant tissues must be utilized (Engelmann 2004). Plant tissues are mostly water. Therefore in order to safely freeze these tissues, they must first be desiccated or otherwise treated to prevent the formation of the most damaging forms of ice crystals.

Very early cryopreservation protocols avoiding freezing tissue damage through very specific step-wise protocols for tissue cooling (Mazur 1984; Engelmann 2004). Tissues would be cooled at a rate of 0.5 – 2.0 °C per min until a predetermined temperature was reached (generally -40 °C). Then samples would be rapidly immersed in liquid nitrogen, to attain the preferred long term storage temperature of -196 °C. Some species are stored at warmer temperatures. For example, Monterey pine (*Pinus radiata*) embryogenic cell lines have been successfully stored at -80 °C for 1 yr, before recovery and germination (Montalbán and Moncaleán 2017) The rapid cooling rate of this procedure allows for the supercooling of plant materials, while the membrane excludes ice crystals which may be forming in the exterior medium. Then, with the plant membrane acting as a semi-permeable barrier, the supercooled nature of the internal cell space

results in increasing aqueous vapor pressure in the cell relative to the exterior space. As a result, water leaves the cell, resulting in lower or absent intracellular water levels before final immersion of the tissues into liquid nitrogen. This method requires an extensive set-up for rapid cooling, and can be difficult to implement with tissue explants consisting of multiple organs, all of which may require different temperature to reach an appropriate stage of supercooling. As a result of these limitations, this protocol is rarely practiced today (Engelmann 2004). Instead, tissues for cryopreservation are pre-treated utilizing a range of vitrification-based protocols. In the context of cryopreservation, vitrifying tissues involves the formation of amorphous ice, as opposed to ice crystals, in tissues placed in below freezing temperatures (Tulk et al. 2002). This amorphous ice is a semi-solid, which does not expand and damage tissues as do ice crystals. In vitrification protocols, tissue explants are still desiccated prior to cryopreservation (Engelmann 2004). However, desiccation is achieved through a range of air-desiccation or medium treatments, many of which have proven to be species-specific (Panta et al. 2015). Encapsulation has been used to aid a desiccation pre-treatment, and then the tissues are stored encapsulated as synthetic seeds for cryopreservation (Wilkinson et al. 2003). Shoot tips of chocolate cosmos (*Cosmos atrosanguineus*) stored in alginate strips demonstrated 100 % survival rates after 12 mo of cryopreservation storage in liquid nitrogen. A pre-treatment medium with very high sucrose content may be used to help remove extracellular water from tissues (Panta et al. 2015). Pre-treatment medium may also contain cryoprotective substances, designed to help protect tissues from freezing damage during cryopreservation. Anti-freeze proteins added to these pre-treatment media greatly increased tissue survival and recovery in *S. tuberosum* and *Chrysanthemum* x *grandiflorum* shoot tips during cryopreservation (Jeon et al. 2015; Seo et al. 2018). As well, material may simply be allowed to desiccate under air flow from filtered compressed air or a laminar hood. Given the recent development of efficient cryopreservation protocols, few studies investigating the utility of cryopreservation in plant conservation have followed their subjects beyond 2 yr, which suggests that cryopreservation as a methodology remains to be proven (Pence et al. 2017). However, Pence et al. (2017) regenerated shoot tips of Todsen's pennyroyal (*Hedeoma todsenii*) after 13 yr in cryopreservation, and found 72.0 % of their cultures survived and were able to continue growth after thawing. However, they did note the presence of possible somaclonal variants among their regenerants.

Although tissue culture conservation techniques have been separated in this review, many tissue culture conservation systems incorporate more than one strategy for a single species. For particularly valuable plant species, many different avenues of tissue manipulation and preservation have been incorporated into the same program. Madagascar periwinkle (*Catharanthus roseus*) is a wild species valued for its potent anticancer properties (Salma et al. 2018). Established protocols have been developed for explant establishment, shoot proliferation, somatic embryogenesis, slow growth, and cryopreservation (Chen et al. 1984; Bakrudeen et al. 2013; Mehta et al. 2013). Storing material through more than one means can help reduce the effect of the unintentional production of somaclonal variants during culture. While tissue culture techniques such as somatic embryogenesis are known to cause variants, other techniques which were previously believed to maintain clonal fidelity have been found to be responsible for the production of variants (Pence et al. 2017). For example, plantlets of cacao (*Theobroma cacao*) regenerated from somatic embryos had a greater proportion of variants if they were first cryopreserved (Adu-Gyamfi et al. 2016).

While tissue culture methodology is important to plant conservation, the use of this technology alone may not save species from extinction. Sufficient genetic diversity must be maintained to prevent genetic drift and inbreeding (Namkoong 1991; Fant et al. 2016). Existing ex situ conservation programs have been found to maintain poor or absent records of the genetic diversity of their populations, with no mechanism in place to prevent inbreeding or ensure that genetic diversity is maintained in cultivated populations across different institutions. As well, a 2010 report noted that some of these critically important ex situ plant collections had been lost over time, likely as a result of inbreeding depression and the ensuing loss of fitness. Govaerts (2010) noted that out of the 844 plant species reported as extinct or extinct in the wild on the IUCN Redlist, 14.0 % had initially been conserved in Botanical Garden collections. However by 2010, 5.0 % of these had been lost over time. For a conservation-orientated program, it is inexcusable to lose protected species through genetic mismanagement, particularly those which are currently extinct in the wild.

*C. dentata* is a functionally extinct and declining plant species in North America, and one where in situ conservation is complicated by human activities and the continued presence of an introduced fungal pathogen that causes high mortality (Van Drunen et al. 2017). Several techniques in tissue culture have the potential to be beneficial to the preservation of this species.

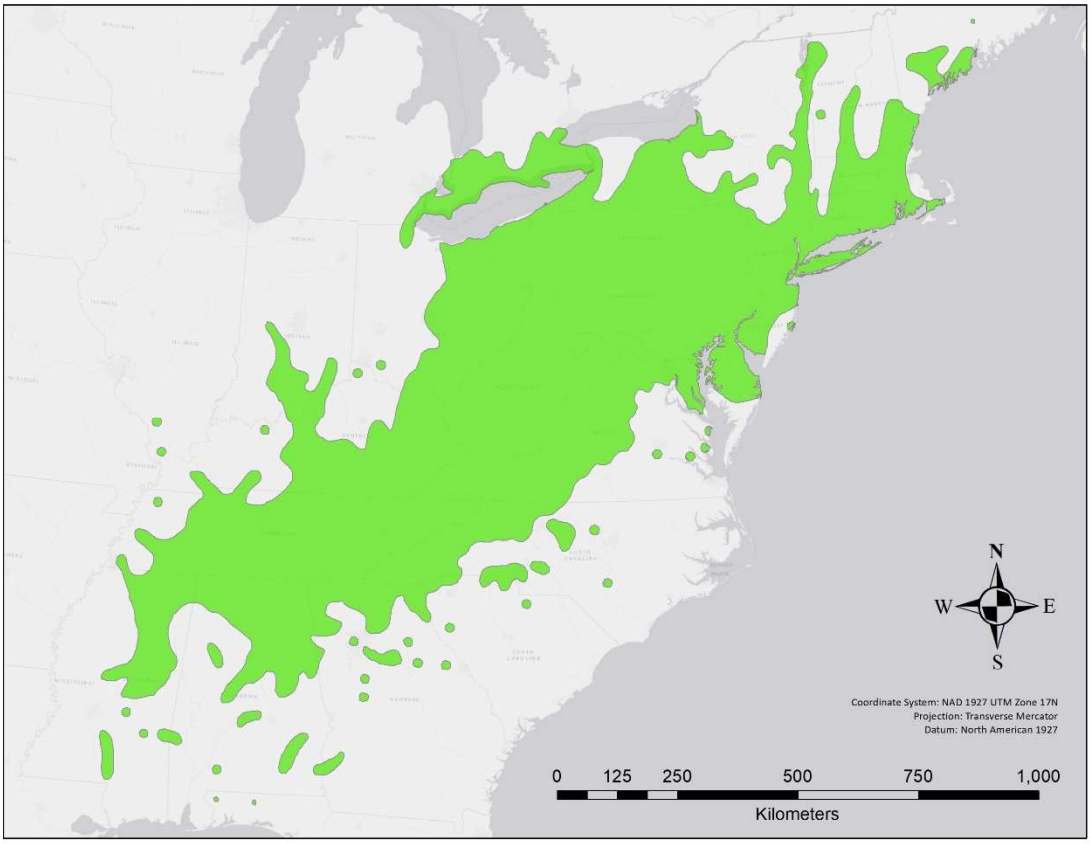
However, conservation work must be carried out with the intention of maximizing genetic diversity over time.

### 2.3. Study species: American chestnut (*Castanea dentata* (Marsh.) Borkh.)

#### 2.3.1. *The genus* Castanea *and* C. dentata

*Castanea* is a genus of fast-growing and long-lived hardwood nut-producing plants in the Fagaceae family (Bolgiano and Novak 2007). The genus evolved and diversified in Asia during the Eocene (Lang et al. 2006; Lang et al. 2007). To date, Asia remains the region of greatest *Castanea* diversity. The European clade of *Castanea* diverged from its common ancestor approximately 42 million years ago (MYA). American *Castanea* species diverged from European ancestors approx. 39 MYA. Pollen records showed that *C. dentata* populations were restricted to south eastern North America as a result of glaciation during the last 18 000 yr (Delcourt and Delcourt 1981; Davis 1983). As glaciers retreated, *C. dentata* moved northward into the range it occupies today. However, given the self-incompatibility and animal-dispersed nature of *C. dentata* nuts, this species is the slowest of the migrating deciduous tree species which followed retreating glaciers. It has even been speculated that *C. dentata* was still migrating northward during the late 19$^{th}$ century. The reported range of *C. dentata* stretched from Northern Florida (USA) to Southern Ontario (Canada) (Figure 2.1). However today, specimens of *C. dentata* are found growing northward of the original range reported in the early 20$^{th}$ century.

**Figure 2.1.** Range map of *C. dentata* (modified from Little 1977; USGS 1999).




*C. dentata* is one of eight identified species in the *Castanea* genus (Lang et al. 2006; Lang et al. 2007). Species within the genus are divided into two clades based on nut production, with individuals producing either one or three nuts per burr (Lang et al. 2007). The three-nut clade includes all commercially harvested nut species; American chestnut (*C. dentata*), Chinese chestnut (*C. mollissima*), European chestnut (*C. sativa*), Japanese chestnut (*C. crenata*), and Dode's chestnut (C. *seguinii*) (Vieitez 1995; Lang et al. 2007). Species in the three-nut clade are generally large trees. Species in the one-nut clade are small trees or shrubs, and include the American chinquapin (*C. pumila*), and the Chinese chinquapin (*C. henryi*) (Lang et al. 2007). *C. pumila* is considered by some to consist of two separate species, tentatively named the Ozark chinquapin (*C. pumila* var. *ozarkensis* or *C. ozarkensis*) and the Allegheny chinquapin (*C. pumila* var. *pumila* or *C. pumila*) (Dane and Hawkins 1999; Lang et al. 2006; Dane et al. 2014). Species in the one-nut clade evolved independently in both Asia and North American approximately 24 – 10 MYA (Lang et al. 2007).

*C. dentata* are found in two architecture types depending on surrounding abiotic and biotic factors. Forest trees are tall self-pruning trunks of up to 40 m in height (Bolgiano and Novak 2007; Wang et al. 2013). Trees grown in open locations do not reach such impressive heights, and instead become rounded orchard-type trees with large full canopies (Freinkel 2007). *C. dentata* is a prolific basal resprouter, and these basal sprouts have contributed to the continued survival of the species in the 20th and 21st centuries (Hawley and Hawes 1925; Paillet 1984; Paillet 2002; Pierson et al. 2007). Leaves are long (20.0 – 30.0 cm), lanceolate with an acuminate apex and deeply serrate margins with a slight hook in some individuals (Vieitez 1995). Stems are reddish and hairless, with alternately spaced buds that hug the stem. Trees are monecious (Zon 1904; Brown and Kirklam 1990). Flowers appear on new year's growth only in late May to early June, and catkins consist of two morphologically distinct forms in *C. dentata*; male unisex catkins, and bisexual catkins containing female and male flowers (Zon 1904; Brown and Kirklam 1990; Sisco and Hebard 2000). Male unisex catkins are very showy, consisting of long, slender clusters of white flowers, and are generally located on the weaker lateral shoots and the lower portions of thicker vertical shoots. Bisexual inflorescences consist of female flowers located at the base of the catkin (close to the stem), and shortened male catkins Bisexual flowers are generally restricted to the upper portions of thicker, upper vertical shoots. Flowers are wind-pollinated, and trees are generally believed to be self-incompatible. *C. dentata* pollen does not appear to travel as far as some wind-dispersed species, and has been measured traveling a maximum of 100.0 m (Russell 1987; Paillet 2002). The mechanism for self-incompatibility has not been identified in *C. dentata* (Davis 1983). However, in the closely related *C. mollissima*, fertilization failure occurs after sperm delivery into the embryo sac (Sage et al. 1994). It is possible that self-incompatibility is regulated in *C. dentata* by a similar mechanism. The seed of *C. dentata* are contained in a dark nut enclosed in an extremely prickly burr (Zon 1904; Saucier 1973). Nuts remain protected inside the burr during development. In September and October, the burrs open and release the mature nuts, which fall to the ground if not eaten by an animal (Webb 1986; Russell 1987; Brown and Kirkman 1990). Three nuts are produced per burr, with nuts ranging in diameter from 1.5 – 4.0 cm. As a commercial product, nuts from *C. dentata* are generally smaller than nuts produced by the other commercial *Castanea* species (*C. sativa, C. crenata,* and *C. mollissima*). However, nuts from *C. dentata* are considered to be the sweetest of all within the 3-nut clade, and are the only *Castanea* nut sweet enough to be eaten raw (Van Fleet 1914; Wang et al. 2013). *C. dentata* was also one of the most

prolific producers of all North American nut species (Wang et al. 2013). Trees growing in open-grown fields were reported to begin nut production at 4 yr old (Paillet and Rutter 1989). Trees under a forest canopy, or subject to considerable interspecific competition, typically began nut production from 8 – 20 yr of age. A mature tree could produce a reported 6000 nuts per year, and did not demonstrate yearly variances in masting behaviour as seen in closely related nut species such as *Quercus* spp. (Zon 1904). Germination was reportedly high, with 60.0 – 70.0 % field germination and > 90.0 % germination under greenhouse conditions (Wang et al. 2006; Clark et al. 2012).

*C. dentata* was a very common and prolific species up until the beginning of the 20$^{th}$ century (Wang et al. 2013). Near the center of its range, it has been reported that forest growth could consist of up to 50.0 % *C. dentata* (Buttrick 1913; Davis 1983; McEwan et al. 2006). However, recent work examining historical land survey data suggests that *C. dentata* abundance never passed 25.0 % for any given region (Faison and Foster 2014). *C. dentata* is believed to be a species characteristic of mature forests. Seedlings grown under shaded canopy conditions tend to stall growth once stem diameter has reached 20.0 cm (Paillet 2002; McEwan et al. 2006). However, if exposed to full light, trees will rapidly initiate new growth and can reach full size (> 40.0 cm at diameter at breast height: DBH) in 30 yr (Paillet and Rutter 1989; McEwan et al. 2006). *C. dentata* trees rapidly gain in stem diameter under optimal conditions; as much as 1.2 cm in diameter a year (Paillet and Rutter 1989; McEwan et al. 2006). This is a greater rate of growth than seen in similar hardwoods such as *Quercus* spp., *U. americana*, Black walnut (*Juglans nigra*), shagbark hickory (*Carya ovata*), and even the relatively fast-growing big-tooth aspen *Populus grandidentata* (Jacob and Severeid 2004; McEwan et al. 2006). When a canopy opening occurred in a mature forest, this potential for rapid growth rate ensured that *C. dentata* was the most likely species to take advantage. Although naturally a climax forest species, it was also reported as being the most common species in secondary forests arising after European disturbance, with *C. dentata* alone accounting for 50.0 % of second growth forest in New Jersey, Pennsylvania, Connecticut, and southern New England (Hough 1878; Frothingham 1912; Russell 1987; Wang et al. 2013). Likely this proliferation was a combined function of its high seed set, fast growth rate, and ability to form basal sprouts (Mattoon 1909; Paillet 2002).

### 2.3.2 History of decline and current restoration efforts

*Cryphonectria parasitica* (Murr.) Barr is an Asian pathogenic fungus and the causal agent of chestnut blight. Analysis of the population genetics of *C. parasitica* and comparison to extant importation records from the 18th and 19th centuries suggests that *C. parasitica* was introduced to North America multiple times through the importation of *C. mollissima* or *C. crenata* (Powell 1900; Anagnostakis 1987; Milgroom et al. 1992; Milgroom et al. 1996; Dutesh et al. 2012). However, blight was first noticed by the scientific community in 1904, when an American chestnut in the New York Botanical Garden was afflicted with an unidentified rapid dieback disease (Merkel 1905; Hepting 1974). The pathogen was initially identified as a new member of the *Endothia* genus, and named *E. parasitica*. A closer examination of morphological characteristics in 1978 led to the reclassification of the species as *Cryphonectria* (Barr 1978; Micales and Stipes 1987). In 1912, after the rapid loss of all 1 500 *C. dentata* trees in the New York Botanical Garden, the newly named "chestnut blight" was formally recognized by the American public and scientific communities (Freinkel 2007). The disease spread rapidly throughout the range of American chestnut. By the end of the 1940s, the blight had reached all corners of its native range. Between this devastating disease, and the ensuing tree harvesting that came about once an awareness of disease presence became known, an estimated three to four billion trees were lost (Paillet 1984; Griffin and Elkins 1986). Today, only a fraction of the original meta-population is extant, and most of this exists in managed seed orchards (Brewer 1995; Steiner et al. 2017).

***Conventional breeding and the TACF***

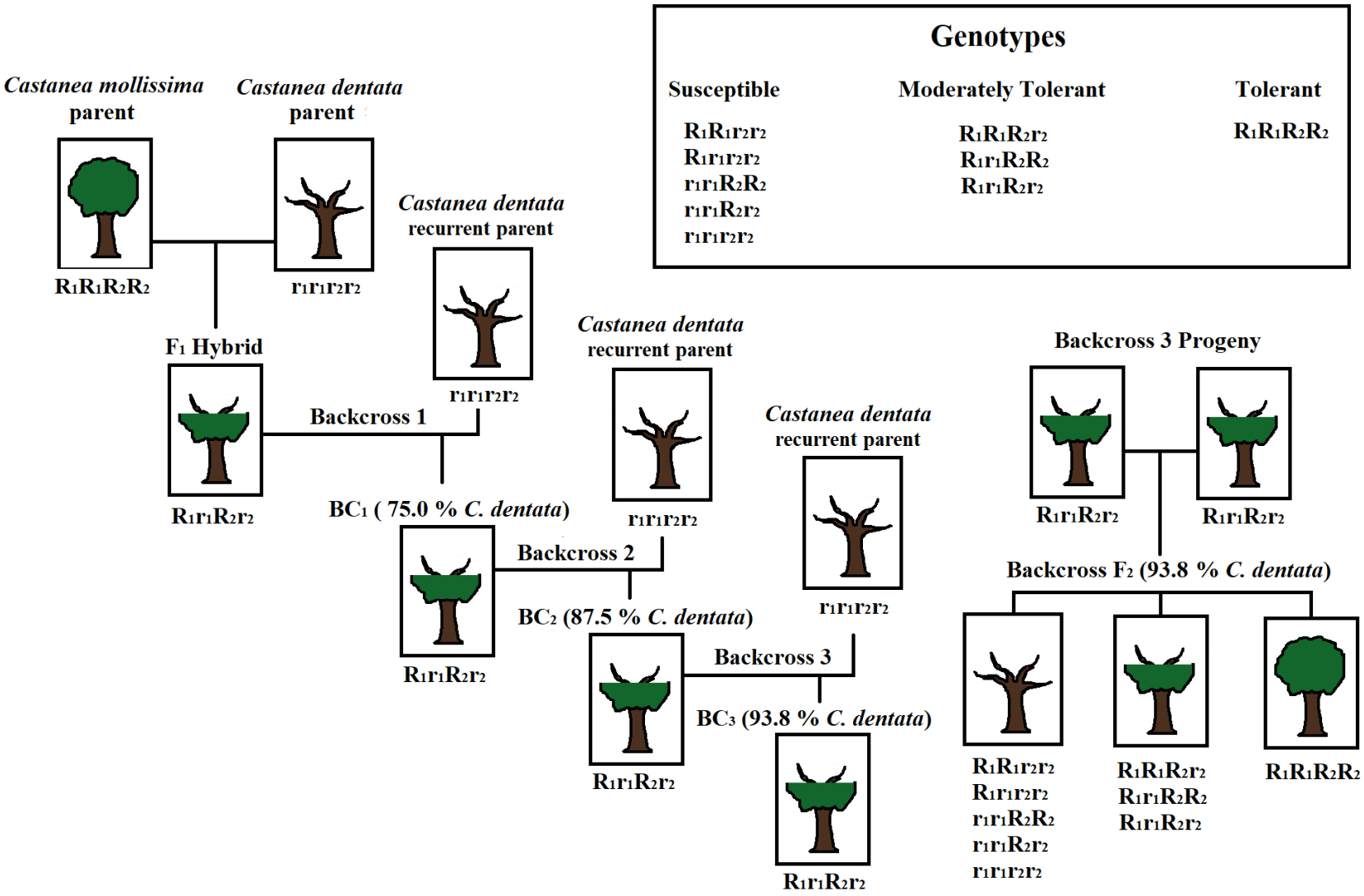
For over 100 yr, conventional breeding techniques have been used in an attempt to promote blight tolerance in *C. dentata* (Diller and Clapper 1965; Steiner et al. 2017). Asian chestnut species are very tolerant to *C. parasitica* (Graves 1950; Dane et al. 2003; Mellano et al. 2012). Although some individuals from Asian species have died from blight infection, many more host the fungus asymptomatically. *Castanea* spp. readily hybridize within the genus (Wang et al. 2013). Even before the blight occurred, *C. dentata* and *C. pumila* were being hybridized with *C. mollissima* and *C. crenata* species to improve various nut production traits in the American species. In response to the blight, many different breeding programs have tried to incorporate the blight tolerance of Asian species into *C. dentata* (Clapper 1952; Burnham 1988; Wang et al. 2013; Steiner at al. 2017). However, for many of the most commercially desirable traits in *C. dentata* (tree architecture, nut quality), Asian *Castanea* species have a very different phenotype. While *C. dentata* is a tall timber-

type tree that produces very sweet nuts, Asian *Castanea* are shorter, shrubbier trees and produce larger and relatively more bitter nuts that require roasting before eating. Therefore, as a timber tree and a nut crop, Asian species are inferior to American chestnut. As well, for much of the period of time that *Castanea* breeding has been undertaken in North America, the mechanism of inheritance for blight tolerance has been all but unknown (Steiner et al. 2017). DNA was only discovered as the unit of heredity in 1928, a mere 3 yr before the first major blight-tolerance breeding programs were initiated (Griffith 1928; Clapper 1952; Wang et al. 2013). Therefore a significant challenge was undertaken by the first early breeders for blight tolerance in *C. dentata*.

There have been two major breeding efforts to achieve a blight tolerant hybrid chestnut. From 1931 to 1960, Arthur Graves (later joined by the USDA) made thousands of *Castanea* crosses in an attempt to produce a blight tolerant and commercially viable hybrid chestnut (Clapper 1952; Wang et al. 2013). Unfortunately, no hybrids were ever produced which met these criteria. Hybrid offspring featured a wide range of phenotype combinations from the two parent species. However, no combination of *C. dentata* phenotypes with the blight tolerance of Asian *Castanea* was ever achieved in a hybrid (Wang et al. 2013). The next major breeding program, one which is still ongoing today, is the backcross breeding program currently maintained by TACF (Burnham et al. 1986; Steiner et al. 2017). Initiated in 1981 by Burnham and Rutter, the TACF backcross program is the longest running backcross breeding program in the world, and involves 16 states in the USA (Clapper 1952; Burnham 1988; Steiner et al. 2017). This backcross breeding program involves breeding through six successive generations (Figure 2.2) to produce $B_3F_3$ lines that would be approx. 93.8 % *C. dentata*, 6.2 % *C. mollissima*, and as blight tolerant as the Asian parent, and finally have all the commercial characteristics of *C. dentata* (Steiner et al. 2017). A minimum of 25 individuals specimens of *C. dentata* are used as parents in each backcross generation, to maintain high levels of genetic diversity in the hybrids (Namkoong 1991; Hebard 2002). This breeding program was built around the assumption that blight resistance in *C. dentata* is found on at least 3 separate loci, with the intention of developing homozygosity for blight tolerance at all three loci (Hebard 2002; Steiner et al. 2017). However, it should be noted that the genetic basis for blight tolerance in *C. dentata*, or any *Castanea* species, is currently not understood. The TACF is testing their first groups of 6-generation $B_3F_3$ hybrids in the field (Jeffers et al. 2011; Steiner et al. 2017). These hybrids are young specimens, and their overall blight tolerance has not yet been effectively demonstrated. Even if a hybrid showed blight tolerance, the polygenic inheritance of

blight tolerance suggests that the hybrids would be unlikely to pass on their blight resistance genes effectively to existing wild populations of *C. dentata*. Homozygous blight tolerance would likely be inherited as heterozygotes, and may be lost in the larger blight-susceptible gene pool found in wild populations, unless heavy selection pressure promotes the fitness of the blight tolerant homozygotes over all allelic profiles.

**Figure 2.2.** Schematic of the backcross breeding program proposed by the TACF (adapted from Burnham 1988).



***Genetic transformation***

In addition to conventional breeding, the TACF has also invested resources into genetically engineering a blight resistant *C. dentata*. Specifically, the New York chapter has been pursuing research along two lines, creating a cis-genetic *C. dentata* with blight resistance genes directly from blight tolerant *C. mollissima*, and engineering a transgenic *C. dentata* with an oxalate oxidase gene from barley (*Hordeum vulgare*) (Polin et al. 2006). Cis-transformation has recently been achieved in the closely related *C. sativa* with a thaumatin-like protein gene (CsTK1) isolated from the cotyledons of seedlings (Corredoira et al. 2012). In *C. dentata,* a limited number of transgenic lines have been engineered with a functioning copy of the oxidase oxalate gene (Zhang et al. 2013; Newhouse et al. 2014). Work with the Forest Health Initiative was also undertaken to improve transformation rates with *C. dentata* (Kong et al. 2014; Nelson et al. 2014). These transformed lines demonstrated very promising results in the lab; 20 lines are currently undergoing field evaluation. Stem assays with blight susceptible *C. dentata*, the transformed *C. dentata*, and blight tolerant *C. mollissima* have indicated that transformed *C. dentata* have even greater blight tolerance than *C. mollissima*. There is also evidence that this trait is heritable (Newhouse et al. 2014). Although there are limited studies examining the host-pathogen interactions which take place during infection between *Castanea* and *C. parasitica*, oxalate produced by the fungus plays a crucial role during infection (see Chapter 5). Oxalate has been implicated in lowering the pH of host tissues to the acidic conditions optimal for the functioning of fungal enzymes, and in some species also works in tandem with polygalacturonases to hydrolyze pectates from the middle lamella of host cells (Bateman and Beer 1965; Griffin and Elkins 1986). The oxalate oxidase gene from wheat deactivates fungal oxalate through conversion of oxalate to carbon dioxide and hydrogen peroxide (Berna and Bernier 1999; Zhang et al. 2013). In transgenic *C. dentata*, oxalate oxidase is overexpressed constitutively in host tissues (Zhang et al. 2013). When *C. parasitica* infection begins, the constitutive oxalate oxidase immediately converts the fungal enzyme to innocuous carbon dioxide and hydrogen peroxide. As a result, infection cannot proceed beyond this point, and the host is successfully able to wall off the infection behind wound periderm.

To date, transgenic trees have performed admirably in the lab and field trials, and have displayed heritable blight tolerance. However, they have yet to pass the greatest hurdle, regulatory approval for public dissemination. *C. dentata* is no longer a commercially relevant species. To release a transgenic American chestnut in the current political and socioeconomic climate would

be for one purpose only, conservation. However, never in the history of transgenics has a genetically modified organism been approved to essentially replace its wild-type counterpart. The approval of the transgenic *C. dentata* for release would set a number of precedents, both for conservation and for the genetic engineering industry. There are several federal regulatory agencies that are involved within the USA (McHughen and Smyth 2008). The Environmental Protection Agency (EPA) traditionally deals with biopesticide crops, such as Bt corn. The Food and Drug Administration (FDA) regulates genetically modified plants and animals consumed by humans. Finally, the United States Department of Agriculture (USDA) further regulates genetically modified crop plants and animals. Transgenic *C. dentata* needs to be examined by one or more of these three agencies. While the argument could be made that these transgenic trees are necessary for the continued survival of *C. dentata* as a native species, there are many concerns with the precedent this release would set. Only time will tell if the transgenic tree will be approved for release in the USA. In Canada, the other country within the native range of *C. dentata*, protection of the species is unfolding differently.

***Restoration of Canadian populations of* C. dentata**

At the turn of the century, there were an estimated 2 million American chestnut (*Castanea dentata* (Marsh.) Borkh.) in Canada (McKeen 1995). Between 2001 and 2003, an extensive survey of the remaining Canadian *C. dentata* trees was conducted throughout its historic range (Tindall et al. 2004). Only 601 wild trees were located; 50.0 % were juveniles and 25.0 % had signs of blight infection. The results of this survey documented an overall population loss of over 99.9 %, and an extant Canadian meta-population with few escapees and little reproductive potential. In 2015, a follow-up survey was done over the same geographic area (Van Drunen et al. 2017). A greater number of trees were located than in the 2004 survey, for a total of 1500 extant individuals. However, few of these were large trees (approx. 80.0 % were < 20.0 cm DBH). In addition, almost 25.0 % of the trees documented in the 2004 survey were deceased. Of the trees with blight symptoms in 2004, 40.0 % died over the past decade. This indicates that 60.0 % of the blight-infected trees survived over the past decade, and may hint that remaining trees are developing some tolerance to the blight as predicted by McKeen (1995). However, blight is only one obstacle to the survival of the American chestnut in Canada. One of the most troubling observations of the 2015 survey was the very low recruitment rate of new trees into the extant Canadian *C. dentata* meta-population. *Castanea* species are self-incompatible. So few *C. dentata* remain in the field

that most extant *C. dentata* populations consist of a single tree (Tindall et al. 2004; Van Drunen et al. 2017). This is a barrier to cross pollination and ultimately nut production. Only two seedlings were observed in the field during the 2015 study (Van Drunen et al. 2017). Although Canadian populations of *C. dentata* may be adapting to the blight, at current rates of recruitment this species will be extinct in Canada by 2100.

The CCC and the Government of Ontario have developed a recovery plan for the American chestnut in Canada (Environment Canada 2016). This plan involves restoring the natural reproductive potential of remaining *C. dentata* populations to above replacement levels. Termed "Breaking Isolation", this ambitious plan involves strategic planting of genetically diverse *C. dentata* trees within pollination range of existing isolated mature trees (Environment Canada 2016; Casier 2018). This would allow existing trees to produce viable nuts, while also greatly increasing the genetic diversity and recruitment rates of remaining populations. In addition to breaking isolation in the native range, the recovery plan calls for the formation of new blight-free plantations of *C. dentata* outside of the native range of Southern Ontario. Plantations outside of the native range will foster assisted migration of the species in response to climate change, and will allow Canadian populations of *C. dentata* to expand in blight-free areas. It is believed that the implementation of this plan will allow *C. dentata* populations to become self-sustaining in Canada within the next decade (Environment Canada 2016; Casier 2018).

### 2.3.3. Chestnut blight (*Cryphonectria parasitica* (Murr.) Barr)

The *Castanea* disease known as Chestnut blight is caused by an Asian endemic necrotrophic ascomycete known as *Cryphonectria parasitica* (Murr.) Barr (Yan et al. 2007). Closely related *Cryphonectria* species (*C. radicalis*, *C. naterciae*, and *C. japonica*) are not pathogenic, but saprophytic on *Castanea* and *Quercus* species (Rigling and Prospero 2018). In its native range, *C. parasitica* is a natural parasite of Asian *Castanea* species. Tolerance to *C. parasitica* varies between Asian species of *Castanea* (Graves 1950; Anagnostakis 1992; Dane et al. 2003; Mellano et al. 2012). As a general rule, *C. parasitica* infection is not fatal to Asian *Castanea* species. *C. parasitica* was introduced to North America on imported Asian chestnut species, likely *C. crenata* and *C. mollissima* (Powell 1900; Anagnostakis 1987; Milgroom et al. 1992; Milgroom et al. 1996; Dutesh et al. 2012). Work on strain diversity has also suggested that the fungus was likely introduced multiple times and in various locations across North America throughout the 1800s (Dutesh et al. 2012). Since its introduction, considerable work has been done

to identify the life history, host-pathogen interactions, and the effects of environment on *C. parasitica*. While more work remains to be done, a better understanding of the pathogen has played a significant role in attempts to control the disease.

***Lifecycle of* C. parasitica**

The fungal parasite *C. parasitica* spreads between hosts through both wind-dispersed ascospores and mechanically dispersed conidia (Sharf and DePalma 1981; Russin et al. 1984; Anagnostakis 1995; Rigling and Propsero 2018). Growth in *C. parasitica* can proceed as soon as external temperatures are above 0 °C, and it can grow effectively at temperatures up to 38 °C (Anderson 1913; Shear and Stevens 1913; Rankin 1914; Stevens 1917; Anagnostakis and Aylor 1984). The germinating spore is not strong enough to break through the periderm of the host, therefore a natural opening is required to infect the tree (Prospero et al. 2006). Mechanical wounding such as graft unions and herbivore damage have been linked to blight infection (Uchida 1977; Hebard et al. 1984; Anagnostakis 1987; Propsero et al. 2006). The age of the wound seems to affect blight infection, and Bazzigher and Schmid (1962) found that wounds older than 4 d were no longer susceptible to blight infection. Infection can also occur naturally through lenticels. Once inside the host, fungal growth is rapid. Periderm tissue is first infected intracellularly by a massive growth of hyphae (Keefer 1914; Bramble 1936). After the accumulation of mycelium has reached a threshold level, flattened mycelium begin to infect the surrounding host tissues through a mycelial-fan (Bramble 1936; Hebard et al. 1984). At the infection edge of the mycelial fan, host cortical and periderm cells are invaded by individual hyphae. During this stage, the pathogen produces fungal oxalate, which reduces the intercellular pH of the host tissues from approx. 5.5 to 2.8 (Havir and Anagnostakis 1983; Griffin and Elkins 1986; Dutton and Evans 1996; Welch et al. 2007). At this reduced pH, optimal conditions occur for the functioning of fungal enzymes and host tissue destruction occurs. Externally on the host, the pathogen produces large masses of visibly noticeable yellow-orange stromata (< 2.5 mm in height) with pycnidia and perithecia, both of which may be visible at the same time (Mortensen 2014; Rigling and Prospero 2018). *C. parasitica* has a mixed mating system, capable of both outcrossing and self-fertilization (Marra et al. 2004). Ascospores are produced in the perithecia. Ascospore production occurs mainly in the fall in North America, and can be seen by 1 mo after infection (Heald and Gardner 1913; Anagnostakis et al. 1998; Robin and Heiniger 2001; Mortensen 2014). Ascospores range in size from 7.0 – 12.0 x 3.5 – 5.0 µm, and are ellipsoidal in shape (EPPO 2005). Conidia are produced

in pycnidia in response to wet conditions, and are mainly disseminated through micro- and macro-fauna (Sharf and DePalma 1981; Russin et al. 1984; Anagnostakis 1995; Mortensen 2014; Rigling and Prospero 2018). Conidia of *C. parasitica* are 3.0 – 5.0 x 1.5 – 2.0 µm, and range in shape from basilliform to ellipsoid (EPPO 2005). There is also some evidence that in the absence of an available host, *C. parasitica* can live for limited periods of time as a saprophyte in the soil (Berry 1951; Burnham 1988; Prospero et al. 2006). *C. parasitica* has even been shown to sporulate while living saprophytically (Prospero et al. 2006). Many tree species outside of the *Castanea* genus, including *Acer* spp., European hornbeam (*Carpinus betulus*), *Quercus* spp., and *Malus* spp., have been documented carrying the blight asymptomatically or with minimal impact (Berry 1951; Diller 1965; Dunn and Boland 1993; Torsello et al. 1994; Dallavalle and Zambonelli 1999; Gryzenhout et al. 2009; Radocz and Tarcali 2009; Tziros et al. 2015). For example, on Hungarian oak (*Quercus frainetto*), *C. parasitica* infection results in slow growing cankers that become callused over (Tziros et al. 2015). As a result of this life cycle flexibility, *C. parasitica* can persist in the environment without a *Castanea* host. Unsurprisingly, once introduced to an area, this pathogen can be found to persist indefinitely except in rare cases where immediate eradication efforts have eliminated fungal material and propagules before dissemination.

***The dsRNA hypovirus and hypovirulence***

In regions of Europe and North America, dsRNA hypoviruses have been identified which naturally parasitizes *C. parasitica* (Grente 1975; Grente and Berthelay-Sauret 1978; Robin and Heiniger 2001; Hillman and Suzuki 2004). These hypoviruses are positive-strand RNA viruses of the family Hypoviridae. They lack a protein coat, and are located solely within host-derived vesicles (Hansen et al. 1985; Fahima et al. 1993). Initially identified in Italian populations of *C. parasitica* on *C. sativa*, at least 4 different dsRNA species of hypovirus, and 6 different strains, have since been identified in Europe and North America (Nuss and Hillman 2011; Feau et al. 2014; Rigling and Prospero 2018). This virus drastically reduces the relative vigor of infected strains of *C. parasitica*. In culture, infected strains grow more slowly than healthy strains, and mycelium color appears washed out or white, compared to the yellow-orange of healthy strains. This virus is of particular importance to *Castanea*, as infected strains of *C. parasitica* have reduced virulence in *Castanea* species. Trees infected with these hypovirulent strains develop only superficial cankers and tree death does not occur (Grente 1975; Burnham 1988).

Mechanistically, the hypovirus has been found to alter as many as 600 different methylation regions and 144 functional genes in *C. parasitica* (Li et al. 2018). Known gene functions impacted include conidial formation, coloration, mating factors, mycelial growth pattern, and alterations to signal transduction pathways involved in the pathogenicity of the fungus (Kim et al. 2002; Anagnostakis 2016; Li et al. 2018). However, many genes known to be impacted by the hypovirus remain to be characterized, and as a result the full impact of the hypovirus on *C. parasitica* remains uncharacterized (Li et al. 2018). However, it is known that infection with the hypovirus results in the reduced expression of several potentially important virulence factors, such as oxalate, laccase, polygalacturonase, cutinase, cellulase, and endothiapepsin (Havir and Anagnostakis 1983; Havir and Anagnostakis 1985; McCarroll and Thor 1985b; Griffin and Elkins 1986; Bennett and Hindal 1989; Hillman et al. 1990; Rigling and Van Alfen 1991; Varley et al. 1992 ; Gao and Shain 1995; Wang and Nuss 1995; Gao et al. 1996; Allen et al. 2003). As well, the hypovirus has been suspected to elicit an increased response in the host, including increased chitinase and salicyclic acid production (Schafleitner and Wilhelm 1997; Schafleitner and Wilhelm 1998; Schafleitner et al. 1999). Finally, one of the primary host defensive mechanisms involves sealing the pathogen behind a wound periderm. It is believed that the slower hyphal growth caused by hypovirus infection is contributing to host tolerance by increasing the time available to form a complete wound periderm (Whitmore 1963; Hebard et al. 1984).

The hypovirus naturally spreads in populations of *C. parasitica*. Fungal colonies arising from conidia also have a 10.0 – 90.0 % chance of also containing the virus (Anagnostakis 1977; Shain and Miller 1992; Enebak et al. 1994; Anagnostakis et al. 1998). However, the virus cannot be spread through ascospores. The virus is mainly spread through hyphal anastomosis; horizontal transfer involving the mixing of cytoplasm following the fusion of hyphae from two strains (Anagnostakis 1977; Zhang and Nuss 2016). However, strains of *C. parasitica* cannot freely undergo anastomosis with any other strain. Anastomosis in *C. parasitica* is limited by vegetative compatibility (VC) types. VC is determined by as many as six defined diallelic genetic loci in *C. parasitica* (termed *vic* loci) (Cortesi and Milgroom 1998; Anagnostakis 2016; Zhang and Nuss 2016). Successful anastomosis can only occur between two strains that share the same alleles at all loci (Anagnostakis 2016). Based on the known number of *vic* loci and the believed genetic basis for successful anastomosis, the current number of VC types in *C. parasitica* is hypothesized to be 64. However, through continued work in the field, a greater number of VC types have been

identified than can be explained by existing knowledge of *vic* loci, and incompatible strains have been found that have identical allelic profiles at the 6 known *vic* loci but do not share the same VC. This suggests that unidentified mechanisms may also be affecting anastomosis between strains of *C. parasitica* (Short et al. 2015; Zhang and Nuss 2016). There is also recent evidence that strains of the same VC type can display different rates of hypovirus transfer, and some VC types may be more resistant to the hypovirus than others, further complicating the role of VC in the spread of the hypovirus (Zamora et al. 2015; Brusini et al. 2017). Clearly, much more is involved in the dsRNA hypovirus and its transmission than is currently understood.

In Europe, a low number of different VC types in the introduced *C. parasitica* population have helped the hypovirus spread naturally in *C. sativa* (Grente and Berthelay-Sauret 1978; Robin et al. 2000; Robin and Heiniger 2001). Currently in Europe, the hypovirus is endemic in much of the natural population of *C. parasitica*, and is responsible for reducing the incidence of mortality from chestnut blight in *C. sativa* to the point where the species is no longer threatened by the disease. Unfortunately, the hypovirus has not been as effective in controlling blight in North America (Anagnostakis 1977). The meta-population of *C. parasitica* in North America contains a much greater diversity of VC types than Europe (Anagnostakis and Waggoner 1981; Robin and Heiniger 2001; Milgroom and Cortesi 2004). This is the result of many different factors. Principally, the differences in diversity of VC types can be linked to a greater number of individuals of Asian *Castanea* spp. imported into North America relative to Europe, as well as misguided intentional inoculations as a form of biocontrol during the early years of disease spread in North America (Shear and Stevens 1917; Hillman and Suzuki 2004). VC types have been found to be very diverse in China and Japan, with nearly 87.0 % of 143 strains tested as having unique VC types. Interestingly, this study also noted that only 3 of the VC types identified from Asian populations were compatible with any of the 65 VC types identified in Europe (Liu and Milgroom 2017). Given the large number of VC types in the native range of *C. parasitica*, it should be expected that a greater number of importations of the host would result in a greater diversity of VC types in North American. However, in North American stands of *C. dentata* where the number of VC types in low, hypovirus inoculations have been proven to be effective in reducing blight related mortality (Davelos and Jarosz 2004; Double et al. 2018). In Michigan (USA), where *C. parasitica* VC type diversity is low enough for natural spread of the hypovirus, demography in *C.*

*dentata* populations infected with hypovirulent blight are reported to be similar to those in blight-free stands (Fulbright et al. 1983; Davelos and Jarosz 2004).

Recent work has been directed towards engineering a strain of *C. parasitica* to be a "super donor" for the hypovirus (Zhang and Nuss 2016). Zhang and Nuss (2016) have been successful in generating a knock-out mutant for four *vic* loci, resulting in a strain which is able to form anastomosis with a wide range of allelic combinations of *vic* alleles. However, recent work has demonstrated that hypovirus transmission may be impacted by more factors than simply VC type. As well, this transformed pathogen has yet to be tested under field conditions. While a promising approach, it remains to be seen if this will be an effective tool for increased hypovirus transmission.

***Disease symptoms in host***

Initial infection manifests in the host as a large stem canker (Mortensen 2014). Stem canker quality will differ between virulent and hypovirulent strains, as well as the age of the host (Heiniger and Rigling 1994). Cankers formed by hypovirulent strains are generally superficial infections of the bark, which either do not reach the vasculature at all or only cause minor necrosis (Griffin et al. 1993). However, recent studies have suggested that cankers caused by hypovirulent strains can sometimes have the same external appearance as the virulent cankers (Bryner et al. 2013). Virulent cankers present in a number of distinct ways. If cambial tissue is killed by the fungus, the canker will appear sunken. However, cankers can also appear swollen. On smaller trees with smooth bark, cankers typically take on a reddish-brown coloration over the canker area. In older trees with a much more pronounced rhytidome, any color change is much more subtle, and indeed cankers on older stems may only be distinguishable once bark begins to split from canker expansion (Diller 1965). Cankers of virulent strains will eventually be covered with a visible layer of yellow-orange stromata (Anderson 1913; Rankin 1914; Stevens 1917). The external canker is the visual manifestation of the wound periderm formed by the host to combat the pathogen (Keefer 1914; Bramble 1936; Hebard et al. 1984). Wound periderm begins formation at the infection edge within 10 days of infection (Hebard et al. 1984). The rate of growth of wound periderm determines the relative blight tolerance of the individual tree (Bramble 1936; Hebard et al. 1984). Completely tolerant trees restrict fungal growth to the outer layers of bark by producing wound periderm faster than the expansion of the mycelial fan. In fully susceptible trees, wound periderm forms slowly or incompletely. In most blight infected trees, healthy wound periderm forms but the pathogen is able to either break through the periderm or outgrow the wound periderm. Once fungal hyphae have

grown beyond the wound periderm, mycelial growth proceeds unchecked throughout the host tissues.

As the disease progresses in the host, leaves and inflorescences begin to senesce. In contrast to the typical loss of foliage preceding winter dormancy, the leaves and inflorescences in blight-induced senescence will not absciss and persist on the tree (Wang et al. 2013). In susceptible trees, infection can lead to the death of all above ground biomass in as little as a year (Bramble 1938; Griffin and Elkins 1986). Death of the above ground biomass is not the end for a mature *C. dentata* tree. A previously healthy, reproductively mature tree may continue to produce sprouts from the base of the stem for many years after the initial blight infection (Paillet 1993). These basal sprouts are particularly vigorous, and if undisturbed by blight will eventually reach the size of the original tree (McEwan et al. 2006). However in most instances, when a tree has lost its aboveground biomass to blight, these basal sprouts will only be able to grow large enough to produce nuts for one to two seasons, before they are infected with blight and die back to the crown. It is currently believed that sporadic nut production by these basal sprouts has been the main force allowing this species to persevere in the wild over the past 100 yr (Wang et al. 2013). However, resprouting capacity is limited. All stumps will eventually run out of the stored resources needed to resprout. In the early 1990s, a study of *C. dentata* stumps in Massachusetts found that only 5.0 % of the actively resprouting *C. dentata* stumps in their study area were from trees alive before the initial blight infection (approximately 60 yr before in their region) (Paillet 1993). The average size of basal sprouts in their study area was no more than 2.0 m tall, and 2.0 cm in diameter, and 90.0 % were formed from stumps of 10.0 cm or less in diameter. This would indicate that in this area, the majority of surviving *C. dentata* (~95.0 %) are from nuts produced after the initial blight infection.

It is encouraging to document wild populations of *C. dentata* which have persisted despite the chestnut blight. Unfortunately, these types of persistent *C. dentata* stands are the exception. In the remaining *C. dentata* meta-population, the majority of extant trees or populations are too small or too reproductively isolated to maintain populations in the wild over the long term. This is particularly an issue in Canada, where reproductive isolation is currently supressing reproductive rates (Van Drunen et al. 2017). When reproductive capacity and source plants are limited, as is clearly the case with *C. dentata*, mass clonal propagation techniques such as tissue culture have great potential value to increase and safe-guard the species from extinction.

### 2.3.4. Tissue culture of *C. dentata*

Micropropagation can potentially create an infinite number of new clonal propagules from a single explant (Corredoira et al. 2017). Several *Castanea* species are important commercial species, and optimized micropropagation systems have been developed and published for these. These include *C. crenata, C. henryi, C. mollissima,* and *C. sativa* (Tetsumura and Yamashita 2004; Corredoira et al. 2005; Hou et al. 2010; Xiong et al. 2018). However, the micropropagation of *C. dentata* is poorly documented. To date, only 12 studies have been published which featured any kind of micropropagation system with *C. dentata*. However, many of these are either simplified proof-of-concept studies with poor reproducibility (Keys and Cech 1982; Serres et al. 1990; Yang et al. 2009), or are studies which involve some form of tissue culture but do not investigate the procedure directly (Polin et al. 2006). Existing studies are divided into axillary shoot culture, somatic embryogenesis, or both. Six studies have focused on axillary shoot culture of *C. dentata*. Three of these studies involved preliminary work that was not continued (Key and Cech 1982; Serres et al. 1990; Yang et al. 2009). Two studies focused solely on optimizing rooting rates ex vitro during Stage IV (Oakes et al. 2013; Oakes et al. 2016). The final study on the axillary shoot culture of *C. dentata* was undertaken by a research group from SUNY-ESF (Xing et al. 1997).

#### 2.3.4.1.Axillary shoot culture in *C. dentata*

***Stage I: culture initiation***

Only three studies, currently over 30 yr old, have fully documented Stage I protocols for axillary shoot culture of *C. dentata* (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997). None of these studies reported their success rates during Stage I. However, the Stage I step of axillary shoot culture has been well documented for *C. sativa* (Vieitez et al. 2007). A description of the Stage I culture of *C. sativa* is included here, as this is the best indicator of what may potentially work with *C. dentata* in culture.

Explant material for the initiation of axillary shoot cultures of *C. sativa* has been derived from both mature and juvenile-phase tissues (Vieitez et al. 2007). Explants consisted of stem cuttings with one or two axillary buds. Juvenile-phase explants were harvested from greenhouse grown seedlings, or excised embryos from nuts germinated in culture and then utilized as explants (Vieitez and Vieitez 1980; Vieitez et al. 1986; Roussos et al. 2016). Juvenile-phase tissue in *C. sativa* was relatively vigorous in culture (Vieitez et al. 2007). For mature-phase tissues, source material which resulted in the most vigorous cultures were derived from forced dormant cuttings

of stump sprouts (Vieitez et al. 1986; Sánchez and Vieitez 1991; Sánchez et al. 1997). Cultures have been initiated from mature-phase crown material, but this was more rarely used and generally showed relatively poor vigor in culture (Sánchez and Vieitez 1991; Gaidamashvili et al. 2017). For example, Gaidamashvili et al (2017) noted that only approx. 20.0 % of their Stage I *C. sativa* explants underwent bud break, the number of new shoots never rose above two, and the average shoot length produced was 2.7 cm. Relative vigor of mature-phase crown material was improved through Stage 0 treatments such as pre-harvest etiolation, or repeated seedling grafting of mature scion wood (Ballester et al. 1989; Sánchez et al. 1997; Giovannelli and Giannini 2000). Explant disinfestation procedures were rarely reported. However, for one procedure, dormant woody cuttings were dipped in 70.0 % ethanol for 30 s, before an 8 – 10 min immersion in sodium hypochlorite solution (0.6 % active chlorine) containing 2 – 3 drops of tween 80 (Vieitez et al. 2007).

Explant sources for axillary shoot culture in *C. dentata* were derived from excised zygotic embryos, or nodal stem cuttings with 2 - 3 axillary buds cut from either seedlings or forced dormant shoots from mature-phase trees (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997). Unfortunately, it is not possible to directly compare relative vigor of these explants as culture conditions varied widely between these studies. Three protocols for tissue disinfestation prior to culture have been published for *C. dentata* (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997). Source material was generally disinfested with a two-step system involving exposure to ethanol for a few sec, and followed by exposure to sodium hypochlorite for several min. Nuts for embryo explants were soaked in 95.0 % ethanol for 1 min, before flame sterilization. The pericarp was peeled, and the embryo axis excised (Keys and Cech 1982). Stem cuttings were disinfested with overnight immersion in 0.1 % Alconox followed by immersion in 5.0 % sodium hypochlorite for 10 min. Serres et al. (1990) disinfested nodal cuttings with a 10 min immersion in 0.5 % sodium hypochlorite, followed by a 30 s immersion in 70.0 % ethanol. Xing et al. (1997) disinfested forced stump sprouts with a 10 min dip in 10 or 100.0 % commercial bleach, or a solution of 1.1 % NaOCl with 2 drops of Tween-20 per 100 ml. In all cases, tissue damage and culture establishment were not noted, so it is not possible to compare the relative success of these procedures.

Stage I induction media varied widely between studies (Table 2.1). The only factor in common between all three studies was the inclusion of BAP as the sole cytokinin and sucrose as the source of carbohydrate, although the concentration of both varied widely between studies

(Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997). Macronutrients, micronutrients, gelling agent (type and concentration), all differed, and two formulations included auxin in the form of NAA or IBA. Given the differences in explant source material, it is not possible to directly compare the relative vigor between these studies, and therefore not possible to assess the relative success of the different Stage I media.

**Table 2.1.** Published Stage I and Stage II media for *C. dentata*.

| Medium Components (L) | Stage I | | | Stage II | | |
|---|---|---|---|---|---|---|
| | Keys and Cech 1982 | Serres et al. 1990 | Xing et al. 1997 | Keys and Cech 1982 | Serres et al. 1990 | Xing et al. 1997 |
| Macronutrients | MS | WPM | MS | MS ½ nitrates | WPM | WPM |
| Micronutrients | MS | WPM | MS | MS ½ nitrates | WPM | NN |
| BAP | 1.0 mg | 1.3 µM | 4.4 µM | 0.1 mg | 0.9 µM | 1.0 µM |
| NAA | | 0.5 µM | | | 0.5 µM | |
| IBA | | | | | | 0.5 µM |
| $CaCl_2$ | | | 6.0 mM | | | 6.0 mM |
| $MgSO_4$ | | | 3.0 mM | | | 3.0 mM |
| Agar | | 6.5 g | | | 6.0 g | |
| Phytagel | | | 5.0 g | | | 5.0 g |
| Phytagar (Gibco) | 6.0 g | | | 6.0 g | | |
| Sucrose | 30.0 g | 20.0 g | 35.0 g | 30.0 g | 20.0 g | 35.0 g |
| Niacin | 0.5 mg | | | 0.5 mg | | |
| Pyridoxine•HCl | 0.5 mg | | | 0.5 mg | | |
| Thiamine•HCl | 0.1 mg | | | 0.1 mg | | |
| myo-inositol | 100.0 mg | | | 100.0 mg | | |
| Sequestrene 330 Fe | | | | | | 10.0 mM |
| pH | 5.5 - 5.6 | 5.6 | 4.5 | 5.5 - 5.6 | 5.6 | 4.5 |

***Stage II: repeating division and subculture***

Stage II media also varied widely across studies (Table 2.1). The basal medium most often used was WPM basal salts, supplemented with vitamins from either WPM or Nitsch and Nitsch (NN; Nitsch and Nitsch 1969; Lloyd and McCown 1980; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). Keys and Cech (1982) utilized a basal medium of Murashige and Skoog (MS) with half strength nitrates (Murashige and Skoog 1962). BAP was always included, ranging in concentration from 0.49 µM to 1.0 µM (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997;

Yang et al. 2009). In addition, Serres et al (1990) added NAA at 0.54 µM. The sugar component was always sucrose, at a range of 20.0 – 35.0 g/L (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). Xing et al. (1997) included supplemental $CaCl_2$ (6.0 mM), $MgSO_4$ (3.0 mM), and Sequestrene 330Fe (10.0 mM). Keys and Cech (1982) added several vitamins, including niacin (0.5 mg/L), pyridoxine•HCl (0.5 mg/L), thiamine•HCl (0.1 mg/L), and myo-inositol (100.0 mg/L). The final element, and one which varied the most between studies, was the gelling agent; phytagel, gellan gum, and various agars have been used. Gelling agents were added at concentrations ranging from 3.5 g/L (gellan gum), to 5.0 g/L (phytagel), 6.0 g/L (phytagar) and 7.0 g/L (unspecified agar) (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). Yang et al. (2009) reportedly tested a range of auxins (IBA, NAA, 2,4-D, and 2,4,5-T) and cytokinins (4-CPPU, K, TDZ, Z). However, the methodology and results of this work were poorly reported, and it is unclear if these growth regulators were tested separately or in combination, and what the relative growth rates were between treatments. As such, this study has not been included in Table 2.1. All media were autoclaved at a pH of 5.5 – 5.6 excluding Xing et al. (1997), where Stage II medium was autoclaved at a pH of 4.5. The multiplication rates of Stage II were not reported in these studies, so it is difficult to directly compare the relative efficiencies of their procedures. More work needs to be done to optimize Stage II axillary micropropagation procedures for *C. dentata*.

***Stage III: ex vitro preparation***

*Castanea* spp. rarely root in culture. Instead, each microcutting formed a large vascularized callus ball (Keys and Cech 1982; Vieitez et al. 2007). Many early axillary shoot culture attempts for *Castanea* spp. involved a Stage III rooting stage.

Of published studies involving the axillary shoot culture of *C. dentata*, most included some form of rooting attempt either during Stage III or Stage IV. Most studies involved a Stage III rooting procedure (Key and Cech 1982; Serres et al. 1990; Xing et al. 1997; Oakes et al. 2013). The Stage III rooting medium generally consisted of the Stage II basal medium (either MS or WPM) at half strength or with nitrates reduced by half, and high IBA concentrations (0.5 µM – 369.0 µM) with low levels or no BAP (0.0 – 0.5 µM). Two procedures also included 2.0 g/L activated charcoal (Xing et al. 1997; Oakes et al. 2013). Key and Cech (1982) reported low rooting rates with their procedure (17.0 %), and Serres et al. (1990) did not report on their success rate except to note that their plantlets were eventually acclimated ex vitro. Oakes et al. (2013) reported

a rooting success rate of 93.0 % in their best treatment combination. However, in a later study published in 2016, they had abandoned their Stage III procedure as plantlets rooted in vitro had low survival rates during Stage IV (rates unreported; Oakes et al. 2016). Xing et al. (1997) had a much more involved Stage III rooting procedure with three separate and sequential Stage III steps. The first involved a medium for shoot elongation, identical to the Stage II medium except for the reduced BAP level (half strength). Plantlets spent 2 – 4 wk in this elongation medium. This was followed by a quick dip in 5.0 or 10.0 mM IBA and culture on half-strength MS basal medium, charcoal at 2.0 g/L, and reduced sucrose (20.0 g/L). After root initiation, plantlets were moved back to the elongation medium for continued root development for 3 wk. The result of this 3 mo procedure was a rooting rate of 50.0 % and a STN rate of 40.0 %. These rooting rates were below optimal, and the loss of plant quality through STN further restricts the usefulness of this procedure. Stage IV survival was not reported, so it was not clear whether rooted plantlets survived transfer to the greenhouse.

Stage III rooting is rarely used in modern axillary shoot culture protocols. This additional Stage is costly in time and skilled manpower. It has been suggested for some species that the roots formed in vitro are morphologically abnormal, with fewer root hairs, an impaired ability to take up nutrients, and some difficulty in adapting ex vitro (Seelye et al. 2003). Most modern axillary shoot culture protocols transfer microcuttings directly from Stage II to Stage IV for rooting and acclimatization. For *C. dentata*, the most recent papers skip Stage III and go from Stage II to Stage IV (Oakes et al. 2016).

***Stage IV: ex vitro establishment***

There is only one report of ex vitro rooting of *C. dentata* (Oakes et al. 2016). However, the ex vitro acclimation procedure was not described and the ultimate survival rate of plantlets ex vitro was not reported. However, rooting ex vitro is commonly practiced with the closely related *C. sativa*, so these studies will be included as a potential model for what may work with *C. dentata*.

Rooting of *C. sativa* shoots ex vitro involved dipping cut microshoots into 0.5 % IBA solution before transfer to a soilless potting medium (Chauvin and Salesses 1988; Vieitez et al. 2007). On average, the rooting rate in this protocol was 80.0 – 95.0 %, with 50.0 – 90.0 % final Stage IV survival, although successful acclimatization after rooting was highly genotype dependant (Chauvin and Salesses 1988; Vieitez et al. 2007).

Oakes et al. (2016) rooted microshoots both in vitro and ex vitro. For their ex vitro procedure, they soaked Jiffy 36.0 mm peat pellets in 5.0 ml/L of Clonex Rooting Solution (IBA 0.5 %; Growth Technology Ltd., Somerset, England), adjusted to pH 5.5. Shoots were cut from the callus ball and dipped in Clonex before planting. Shoots were the covered and incubated at 22 °C for 16 h at 31 $\mu mols^{-1}m^{-2}$. A reported 85.0 – 89.0 % rooting occurred after 9 wk. However, plantlets were not followed through acclimation to lower RH. Given the initially high success rates reported in 2013, followed by apparent failure to acclimate as reported in 2016, it is not clear if their 2016 procedure is ultimately a more successful Stage IV protocol.

**2.3.4.2. Somatic embryogenesis in *C. dentata***

Only nine studies have been published over the past 23 yr which feature the somatic embryogenesis of *C. dentata* (Merkle et al. 1991; Xing et al. 1999; Robichaud et al. 2004; Andrade and Merkle 2005; Polin et al. 2006; Johnson et al. 2007; Andrade et al. 2009; Zhang et al. 2013; Kong et al. 2014). Many of these feature somatic embryogenesis as part of a larger research objective, and do not modify existing protocols (Polin et al. 2006; Andrade et al. 2009; Zhang et al. 2013; Kong et al. 2014). All of these studies were conducted by only two research groups; at SUNY-ESF and the Warnell School of Forestry and Natural Resources of the University of Georgia in Athens (USA). Most of the work reported by the SUNY-ESF group has been based on techniques first optimized at Warnell (Merkle et al. 1991; Polin et al. 2006). A general lack of resources and a low overall successful induction rate (0.02 – 3.00 %) has held back innovation in developing optimal somatic embryogenesis techniques for *C. dentata*, as currently all reports describe a slightly modified procedure developed in the very first study published on the somatic embryogenesis of *C. dentata* (Merkle et al. 1991).

***Stage I: culture initiation and initial callusing***

Both Warnell and SUNY-ESF have historically used immature zygotic embryos as explants for somatic embryogenesis (Merkle et al. 1991; Robichaud et al. 2004; Andrade and Merkle 2005; Polin et al. 2006). However, only immature zygotic embryos harvested during wk 6 and 7 post-anthesis generated somatic embryos (Merkle et al. 1991; Polin et al. 2006). Zygotic embryos harvested before or after this 2 wk period failed to form somatic embryos (Merkle et al. 1991). As well, only explants of immature embryos less than 5.0 mm in length or cotyledons smaller than 6.0 $mm^2$ produced somatic embryos (Carraway and Merkle 1997). Even with optimal explant size and harvesting period, only a maximum of 3.0 % of explants developed somatic

embryos in culture (Merkle et al. 1991; Polin et al. 2006; Johnson et al. 2007). This is an extremely low success rate for an explant source. For the closely related *C. sativa*, induction rates from explants of immature zygotic embryos was 10.5 - 57.5 %, which was substantially better than reported with *C. dentata* (Ballester et al. 2001).

Vegetative tissues have been used successfully in *C. sativa* to generate somatic embryos (Ballester at al. 2001; Corredoira et al. 2003; Corredoira et al. 2005). Leaf microcuttings from axillary shoot cultures callused and grew somatic embryos at a rate of 39.0 % (Corredoira et al. 2003). Leaf explants from axillary shoot cultures have several advantages over immature zygotic embryo explants; they are available for harvest throughout the year and do not require the harvesting and loss of a reproductive unit (seed). Leaf explants can also potentially be taken from mature tissues. This allows for the culture and clonal propagation of select individuals with described genotypes, including suspected moderately blight-tolerant *C. dentata*.

***Stage II: embryo formation and growth***

WPM is the standard basal medium used for *C. dentata* somatic embryo culture. In the closely related *C. sativa*, MS basal medium is typically used (Corredoira et al. 2005; Vieitez et al. 2007). With *C. dentata*, the gelling agent used ranged greatly across studies, from 8.0 g/L Phytagar (Gibco) to 3.0 - 3.5 g/L gellan gum (Gel-gro; Merkle et al. 1991; Carraway and Merkle 1997; Robichaud et al. 2004; Andrade and Merkle 2005; Johnson et al. 2007; Andrade et al. 2009).

A wide range of growth regulators have been used with *C. dentata*, and several key observations were made. The production of somatic embryos on immature zygotic embryo tissues of *C. dentata* was apparently auxin-sensitive. Culturing explants on medium containing NAA produced only proembryogenic masses (Merkle et al. 1991; Carraway and Merkle 1997). Indole-3-acetic acid (IAA) induced embryos, but these did not progress past the early cotyledonary stages and did not develop into plantlets. 4-amino-3,5,6-trichloropicolinic acid (picloram) stimulated the growth of somatic embryos in *C. dentata*, and these developed into plantlets (Johnson et al. 2007). However, 2,4-D was the most effective in promoting growth of somatic embryos that could germinate and maintain their embryogenic potential over the long-term (Merkle et al. 1991; Carraway and Merkle 1997; Andrade and Merkle 2005; Johnson et al. 2007; Andrade et al. 2009). The concentration of 2,4-D was critical for embryo development (Johnson et al. 2007). Johnson et al. (2007) found 2.0 mg/L to be the optimal concentration, while 4.0 mg/L was excessive and reduced embryo production. TDZ has been used to stimulate the growth of somatic embryos in

several recalcitrant woody plant species (Hutteman and Preece 1993). However, 0.1 mg/L of TDZ did not stimulate growth of somatic embryos in *C. dentata* (Carraway and Merkle 1997). It is possible that the concentration of TDZ tested in this study was too large, as nanomolar amounts of TDZ have generally been used for other species (Nassar et al. 2011). BAP is the only cytokinin which has been shown to stimulate callus and embryo production in *C. dentata* explants (Merkle et al. 1991; Carraway and Merkle 1997; Andrade and Merkle 2005; Andrade et al. 2009).

***Stage III: embryo germination***

Stage III protocols involve changes to the medium, and in some studies, modifications to abiotic culturing conditions (Carraway and Merkle 1997; Xing et al. 1999; Robichaud et al. 2004; Andrade and Merkle 2005; Johnson et al. 2007; Andrade et al. 2009). Xing et al. (1999) relied solely on changes to the medium for maturation and germination of embryos. The maturation medium was similar to Stage II medium. However, the sucrose concentration was increased from 20.0 to 60.0 g/L. The embryo germination medium was identical to the Stage II axillary shoot culture medium from Xing et al. (1997). Germination medium utilized by the Warnell group generally differed from their Stage II medium only in the addition of 5.0 g/L of activated charcoal (Carraway and Merkle 1997; Andrade and Merkle 2005; Johnson et al. 2007). However, one study did find that 25.0 mM L-asparagine in the Stage III medium did increase embryo germination rate and root length in growing plantlets (Robichaud et al. 2004). For the studies conducted by the Warnell group, a chilling treatment was seen as necessary for somatic embryo germination (Robichaud et al. 2004; Andrade and Merkle 2005; Johnson et al. 2007).

Exposing cotyledonary stage somatic embryos of *C. dentata* to a cold treatment of 4 °C for from 12 – 18 wk increased embryo germination rates from 0.0 % to 51.0 % (depending on the genotype) (Carraway and Merkle 1997; Andrade and Merkle 2005; Johnson et al. 2007). Chilling caused an increase in endogenous levels of $GA_3$ in dormant seeds, which ultimately promoted germination (Deng and Cornu 1992). It is possible that an application of $GA_3$ to the medium used for embryo germination may also have a positive effect on embryo germination, although this has yet to be tested.

The most recent study examining the protocol of somatic embryogenesis in *C. dentata* also examined the effect of light exposure on the maturation of somatic embryos. Specific wavelengths of light induced embryo germination in some recalcitrant tree species (Johnson et al. 2007). Exposing *C. dentata* somatic embryos to red light before and during germination improved

germination rates by 37.8 % over control plants grown in white light. Unfortunately, few other germination treatments have been tried with *C. dentata*.

Tissue culture is a flexible tool for conservation, the potential of which is just starting to be explored. *C. dentata*, as a functionally extinct self-incompatible species with difficulties propagating clonally through conventional means, is an excellent candidate for the use of tissue culture conservation methods. Before medium term or long term preservation methods can be implemented in the conservation of a species, some form of short term conservation method (such as axillary shoot culture or somatic embryogenesis) must be defined and established for the species (Corredoira et al. 2017). Protocols for the axillary shoot culture and somatic embryogenesis of *C. dentata* have been published (Cech and Keys 1982; Serres et al. 1990; Merkle et al. 1991; Xing et al. 1997; Andrade and Merkle 2005). However, the existing methodologies outlined in the literature feature unreported success rates (axillary shoot culture), significant physiological issues (STN), or very low success rates (0.02 - 3.00 % embryo production per explant in somatic embryogenesis). As well, not enough is known concerning the metabolomics the host-pathogen interaction of *C. parasitica* and *C. dentata* for the effective targeting of virulence factors in any breeding program for increased host tolerance. The conservation of this species would greatly benefit from the development of improved methods for axillary shoot culture and somatic embryogenesis, as well as a clearer understanding of the host-pathogen relationship between the introduced pathogen (*C. parasitica*) causing the decline of *C. dentata*, and host defences present in *Castanea* as a genus.

## CONNECTING STATEMENT I

Axillary shoot culture is an effective short term conservation method which is likely to see expanding use in the 21st century (Corredoira et al. 2017). In particular, axillary shoot culture is useful for the rapid clonal propagation of species which do not readily produce seed, rare species, and species which cannot be clonally propagated by conventional means. The development of an axillary shoot culture system is also often necessary for the implementation of medium term or long term preservation tissue culture methods.

*C. dentata* is a declining native nut tree species which does not readily root from cuttings, and has low grafting success (Galic et al. 2014; Galic 2016). The development of an efficient axillary shoot culture system for this species, such as already exists in the closely related *C. sativa*, would be highly beneficial to conservation efforts with *C. dentata*. Existing axillary shoot culture protocols for *C. dentata* have either unreported multiplication rates, or difficulties that include physiological issues such as STN (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). In this study, several strategies were investigated to improve the axillary shoot culture of *C. dentata*. Growth stage of the source material was investigated for its effect on multiplication rate in culture. Incubation temperature was compared across a range of genotypes to determine the effects on multiplication rate. The range in multiplication rates across 22 genotypes from genetically diverse sub-populations was documented (Husband 2016; Gailing and Nelson 2017). Assessments were made concerning which factors (including incubation temperature and medium components) vary in their response across genotype, and axillary shoot culture protocols were optimized at the genotype level. Finally, components in the Stage II medium were examined for their effect on STN. The outcome of this work was the improved axillary shoot culture for the 22 genotypes included in this study, and the formation of a framework for developing optimized axillary shoot culture procedures for other genotypes of *C. dentata*.

# CHAPTER 3

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## Optimizing axillary shoot culture as a short term conservation method for American chestnut (*Castanea dentata* (Marsh.) Borkh.)

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### 3.1. Abstract

Protocols were investigated across 22 genotypes to maximize axillary shoot multiplication rates at the genotype level for short term conservation, and to reduce the incidence of shoot tip necrosis (STN). Factors investigated included explant growth stage (embryo-, seedling-, and mature- stage) of the source material and two culture incubation temperatures (21 ± 1 °C and 31 ± 1 °C). Multiplication rate was measured across 22 genotypes. The role of 6-benzylaminopurine (BAP; 0.5, 1.0, 2.0, 4.0 µM), calcium (3.0 and 6.0 mM), boron (0.025 mM), magnesium (3.0 mM), and gelling agent (agar: 6.0, 7.0, 8.0 g/L, gellan gum: 3.0, 3.5, 4.0 g/L) on STN was investigated. Genotypes derived from 4 mo old seedlings had the greatest overall multiplication rate (7.87 ± 0.38/6 wk culture cycle). Temperature affected multiplication rate in three of eight genotypes tested, with multiplication rate greater at 21 ± 1 °C for one genotype and greater at 31 ± 1 °C for two genotypes. Multiplication rate differed significantly among genotypes and ranged from 1.45 ×/12 wk to 13.25 ×/6 wk. Incidence of STN was decreased to 1.40 ± 0.02 % at 4.0 µM BAP.

Keywords: chestnut blight, *Cryphonectria parasitica*, genotype, shoot tip necrosis, temperature

## 3.2. Introduction

***Short term conservation through axillary shoot culture***

*Castanea dentata* (Marsh.) Borkh was once a dominant tree species on the east coast of North America (Van Drunen et al. 2017). However, an introduced fungal pathogen (*Cryphonectria parasitica* (Murrill.) Barr.) has decimated this species. Numbers have declined by an estimated 90.0 - 99.9 % since the turn of the 20$^{th}$ century, and many individuals persist as resprouting stumps or as juvenile individuals (Dalgeish et al. 2015; Van Drunen et al. 2017). In Canada, recruitment rates in the Canadian meta-population in southern Ontario have fallen below levels needed to sustain the species, and this sub-population may face extinction in the coming century unless measures are taken to reverse this decline (Van Drunen et al. 2017).

The immediate conservation of *C. dentata* requires the efficient clonal propagation of existing select trees with suspected blight tolerance (Van Drunen et al. 2017). However, *C. dentata* cannot be clonally propagated efficiently by conventional means. *Castanea dentata* cuttings rarely form roots, and the species has a low graft success rate over the long term (Vieitez et al. 1987; Galic et al. 2014; Galic 2016). Trees are self-infertile, and many exist in the field as reproductively isolated individuals not accessible for hand-pollination due to size at maturity and location (Van Drunen et al. 2017). The most recent survey of the Canadian sub-population reported that only 11 % of extant individuals were reproductive. The genetic basis of blight tolerance is not currently understood (Steiner et al. 2017). As such, it is not possible to reliably produce offspring with the blight tolerant phenotype of the parent. Several preliminary studies have explored the option of utilizing axillary shoot culture in the clonal propagation of *C. dentata* (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997). However, none have examined axillary shoot culture as a conservation tool in maintaining and propagating select genotypes of *C. dentata*.

Short term conservation methods involve the use of tissue culture in maintaining plants for rapid multiplication or breeding (Corredoira et al. 2017). Duration of short term conservation techniques typically involve tissue manipulation on the order of weeks to months. Of the techniques used in short term germplasm conservation, axillary shoot culture is considered a preferred method for rapid multiplication with high clonal fidelity. Commercially, axillary shoot culture protocols are generally directed towards optimizing multiplication rates at the species level, resulting in the greatest multiplication rate for the majority of selections or cultivars of one species

(Shirin and Mishra 2016). Recalcitrant lines or cultivars which do not propagate well in the optimized protocol are often discarded unless they are of superior commercial value.

For the purposes of conserving declining species, tissue culture must be approached differently (González-Arnao et al. 2017). Protocols must be designed to optimize multiplication rate at the level of individual genotypes, rather than of the species. Variation in the performance of genotypes in culture reflects some of the natural genetic diversity present in a species (Keyes et al. 1980). By optimizing culture procedures at the species level, diversity is minimized. By developing protocols which optimize multiplication at the level of the individual, the degree of genetic diversity in culture is elevated. Studies to date featuring the axillary shoot culture of *C. dentata* have included only a limited number of genotypes (as few as two) or utilized lines derived from the same tree (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). As such, existing methodologies are not designed to incorporate variability across genotypes, and protocols are optimized only for a limited pool of often closely related genotypes.

***Multiplication rate***

Few factors have been investigated for their impact on multiplication rate in axillary shoot cultures of *C. dentata* (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). The growth stage of the source material used to initiate culture significantly effects culture response and multiplication rates in some species (McCown 2000). In published protocols of *C. dentata* axillary shoot culture, explants have been derived from excised zygotic embryos, young seedling tissues, and forced mature-phase dormant crown cuttings (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). However, rates of vigor between these explant types have not been compared.

Cultures of temperate species are generally incubated at room temperature (approx. 21 °C). However, in studies where the effect of temperature on growth in culture was investigated, differences in temperature optima for growth were found to vary across genotypes (García-Mendiguren et al. 2016). As well, some of the temperature optima for growth measured in culture were found to differ significantly from the measured temperature optima of the source plants. For example, regenerated plantlets of Norway spruce (*Picea abies*) had temperature optima ranging from above 28.0 °C to below 18.0 °C (the maximum range tested in this study; Kvaalen and Johnsen 2007). However, the source plants for the initial explants had an optimal range from 24.4 - 25.6 °C. This suggests that the temperature optima for growth of the source plant does not

necessarily predict temperature optima for multiplication in culture. The impact of incubation temperature on the multiplication rate of *C. dentata* has not been investigated in previous axillary shoot multiplication studies. The optimum culture incubation temperature to favor multiplication in culture is not known, nor whether there is any relation to the isotherm of origin of the source population.

***Incidence of shoot tip necrosis***

STN is an ongoing issue for axillary shoot culture of *C. dentata* (Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). Xing et al. (1997) reduced STN rates in *C. dentata* axillary shoot cultures during Stage IV ex vitro rooting from 86.0 % to 23.0 % through the inclusion of an additional 3 wk (Stage III) culture cycle for microshoots in a medium containing BAP (0.89 µM) prior to rooting. However, the underlying mechanism responsible for STN in *C. dentata* was not determined, and STN was not eliminated in the microshoots.

Many causes have been suggested for STN. However, most can be linked to nutrient or growth regulator deficiencies at the apical shoot tip (Abdulnour et al. 2000; Bairu et al. 2009). Deficiencies can be linked to suboptimal exogenous medium components, or impaired uptake as a function of low transpiration rates in culture (George and de Klerk 2008). Exogenous cytokinin content has been linked to STN incidence, particularly in species that are recalcitrant to any form of adventitious root formation in culture (Bairu et al. 2009). Alternatively, STN may occur only after an initial delay, when exogenous medium cytokinins are exhausted and endogenous cytokinin synthesis can no longer meet the biological needs of the developing shoots. For some species, where STN is mainly an issue during ex vitro rooting, dipping the apical tip of the plantlet into a cytokinin solution prior to rooting reduced the incidence of STN (Piagnani et al. 1996). However, such treatments can reduce overall rooting rates in some species (Vieitez et al. 1989). Boron (B), calcium (Ca), and magnesium (Mg) deficiencies or toxicities have been linked to the incidence of STN in many species (Bairu et al. 2009). In some species, B and Ca uptake are interrelated (Miwa and Fujiwara 2010). Gelling agent, both type and concentration, has also been linked to the incidence of STN (Singha et al. 1990). Different gelling agents are composed of structurally different polysaccharide compounds and varying mineral impurities, which can impact mineral and cytokinin uptake (Bornman and Vogelman 1984; Scherer et al. 1988).

The objective of this study was to optimize axillary shoot culture for 22 genotypes of *C. dentata* from Canada and the northeastern United States. Multiplication rates of *C. dentata* in

axillary shoot cultures were improved by optimizing the choice of growth stage of the initial explant (embryo-stage, seedling-stage, or mature-stage) and incubation temperature (21 ± 1 °C or 31 ± 1 °C) for specific genotypes of *C. dentata*. Rates of STN were reduced by optimizing concentrations of hormones (BAP), minerals (Ca, B, Mg, and combinations of Ca/Mg and Ca/B), and gelling agent (agar and gellan gum) in the media. This work can contribute to the utility of axillary shoot culture for short term conservation of *C. dentata*, and provide a framework for axillary shoot culture programs for other endangered species with wide isotherm ranges.

### 3.3. Materials and methods

#### *Culture induction and maintenance*

Source plants were obtained from genetically diverse populations distributed from New York (USA) and southern Ontario (Canada), (Husband 2016; Gailing and Nelson 2017; Van Drunen et al. 2017) (Table 3.1). Genotypes were also included from genetically uncharacterized populations in Nova Scotia (Canada) and Michigan (USA). Despite the wide geographic range, source plants experienced a similar average temperature of 4 – 10 °C (Lovat 2013; Lovat 2019). Explants were collected from two juvenile phase (embryo- and seedling-stage) and three mature (grafts of mature-phase scions on seedling rootstocks, and forced dormant cuttings derived from either stump sprouts or crown cuttings) tissues.

Genotypes derived from immature zygotic embryos were produced according to the procedure defined in Merkle et al. (1991). These were received and maintained as Stage II axillary shoot cultures (in vitro microshoots initiated from nodal cuttings).

Seeds were received from multiple sources and cold-moist stratified at 4 °C for 4 mo prior to planting into a growth chamber. Seedlings were maintained at 21 ± 1 °C at 100 µmol $m^{-2}s^{-1}$ cool white fluorescent light on a 16/8 h day/night cycle. Genotypes AcGraft and ASH1 were derived from seeds from trees originally planted from genetic stock brought up from the USA by British Loyalists who came to Canada in the late 1700s. Therefore, although these genotypes are derived from trees found in Canada, they are not an original part of the Canadian meta-population of *C. dentata*.

Grafts of mature-phase scions on seedling rootstocks were produced from select named trees from the Canadian meta-population of *C. dentata*, valued for phenotypes including potential blight tolerance and self-compatibility (D. Galic (personal communication, 2017); Van Drunen et al. 2017). Grafted plants were also maintained in growth chambers.

Source material for both stump sprouts and crown cuttings were collected as dormant cuttings from mature-phase trees, and leafed out under aseptic conditions as defined by Preece and Read (2007) and Oakes (2015).

Genotypes were maintained on Chestnut Axillary Medium (CAM), modified significantly from Xing et al. (1997). The CAM consisted of Lloyd and McCown's Woody Plant Medium basal salt medium with Nitsch and Nitsch vitamins, 30.0 g/L sucrose, 1.0 g/L casein hydrolysate, 6.5 g/L agar (*Gelidium* sp.), and 1.0 µM BAP, adjusted to pH 5.6 (Nitsch and Nitsch 1969; Lloyd and McCown 1980). Medium components were obtained from PhytoTechnology Laboratories (Lenexa, Kansas, USA), excluding sucrose. Sucrose was Redpath granulated sugar. All cultures were maintained in Magenta© GA7 containers on 80 mL of medium. Stage I cultures consisted of a single explant per container. Repeated subcultures during Stage II consisted of 5 microcuttings per container. Plantlets were maintained at 21 ± 1 °C at 60 µmol $m^{-2}s^{-1}$ for 16/8 h day/night cycle and subcultured every 6 wk. A minority of genotypes (664 and Zrite), required special consideration to account for slow growth. After 6 wk in culture, shoots of genotypes 664 and Zrite would begin to decline without producing enough shoot growth to replace the material used to make the microcuttings. For these recalcitrant genotypes, the clumps of microshoots and vascularized callus were subcultured without subdivision at 6 wk. After 12 wk, shoots were then tall enough for multiplication. For Zrite and 664, multiplication rate was measured every 12 wk (at the end of the second subculture cycle). Based on results reported in this study, the multiplication rates for the Mettler and New 11 genotypes were measured at an incubation temperature of 31 ± 1 °C. All other genotypes were incubated at 21 ± 1 °C. Genotypes used in this study were maintained though a minimum of 3 subcultures in CAM in Stage II before use in any trial. A summary of experimental design can be seen in Table 2. Microshoots generated in this study were successfully transferred ex vitro utilizing the protocol outlined in Lovat (2019).

***Multiplication rate***

For all studies examining multiplication rate, overall growth was assessed by measuring microshoot height, the number of new microshoots produced per microcutting (microshoot production rate), the incidence of STN as measured as a percentage of the total number of microshoots (% STN), and the number of potential microcuttings from each microshoot (multiplication rate) (Table 2). Microshoot height, microshoot production rate, and % STN are measures of culture quality. Multiplication rate is the measure of overall propagation. A potential

microcutting was defined as a stem section ranging between 0.5 - 1.6 cm in length, consisting of 1-3 nodes with a minimum of one axillary bud and one leaf.

**Growth stage of source material**

For the purposes of comparing the effect on growth stage of the source material utilized for explants on multiplication rate, genotypes in this study were classified into one of four explant groups: embryo- (Alessi, Ellis1, and Zoar) , seedling- (AcGraft, ASH1, New 11, New 15, New 16, New 17, and New 19), mature-stage tissue (Dundas, Hamilton Drive, IIPP, LCEM, Light Cemetery, Mettler, Siebenmorgen, Ward, and 664), and not assessed. The genotypes not assessed were those lacking replication within their explant growth stage, including Deacon #2, #5, and Zrite. It is important to note that genotype is a confounding factor in this design. It would have been optimal to be able to extract embryo-, juvenile-, and mature-stage tissues from the same genotype. However, for a heterozygous species with documented low rates of successful somatic embryogenesis (0.02 – 3.00%) such as *C. dentata*, this would not be possible (Andrade and Merkle 2005). To account for this limitation, as many different genotypes representing each growth stage were included as was possible.

**Genotype variability in multiplication rate**

Through preliminary work, 6 wk was determined to be the period of maximum growth for the majority of genotypes of *C. dentata*. As such, multiplication rate was measured at 6 wk for the majority of genotypes, although some genotypes, such as Mettler, could have been subcultured more frequently due to a faster growth rate (Table 2).

**Incubation temperature and isotherm of origin**

Multiplication rates were determined across two temperature levels (21°C ± 1 and 31°C ± 1) for eight genotypes (Table 2). Temperature regimes were chosen based on earlier studies of temperature ranges for micropropagation (de Capite 1955). Genotypes Dundas, Hamilton Drive, Light Cemetery, Mettler, New 11, New 15, New 16, and New 17 were used. All genotypes examined were obtained from similar isotherms (average isotherm of 4 – 10 °C; Table 3.1).

***Incidence of STN***

**BAP**

The BAP treatments were derived from the concentration of 0.89 µM utilized in Xing et al.'s 1997 study of STN in *C. dentata*. Five levels of BAP were examined (Table 3.2). The genotypes AcGraft, ASH1, and New 17 were used in this trial.

**Ca, Mg, and B**

Treatments for Ca, Mg and B trials were based on previous research into STN in *C. sativa*, *C. dentata*, and recommended treatment regimens to identify the source of STN in other plant species (Table 3.2) (Piagnani et al. 1996; Xing et al. 1997; Abdulnour et al. 2000). Both Ca and B were investigated separately, before examination in combined treatments. Four Ca treatment levels, were used; one from Xing et al. (1997) which included additional Mg. Ca was added as $CaCl_2$, and Mg in the form of $MgSO_4$. For this trial, the genotypes Light Cemetery, New 16, and New 17 were used. The B was used at five levels and added in the form of $H_3BO_3$. The genotypes AcGraft, ASH1, and New 17 were used for the B trials. The combination Ca and B treatment concentrations were taken from studies with *C. dentata* and potato (*Solanum tuberosum*), and suggested concentrations of B for facilitating Ca uptake. Six Ca/B treatment levels were tested (Xing et al. 1997; Abdulnour et al. 2000). For this trial, five genotypes were examined, including AcGraft, ASH1, LCEM, New 11, and New 17.

**Gelling agent**

A comparison of gelling agents was based on studies involving *C. dentata* and *C. sativa* (Table 3.2) (Xing et al. 1997; Andrade and Merkle 2005; Corredoira et al. 2015). Agents investigated were agar (derived from *Gelidium* sp., gel strength 910 g /cm$^2$; PhytoTechnology Laboratories) and gellan gum (derived from *Sphingomonas elodea*, gel strength > 800 g/cm$^2$; PhytoTechnology Laboratories). Genotype AcGraft, ASH1, and Mettler were examined in this study.

***Statistical Analysis***

Culture vessels were arranged in a completely randomized design (CRD) within the growth chamber to minimize the impact of possible abiotic gradients during incubation (Compton et al. 2000). All experiments were replicated a minimum of three times, replicated over time (Table 3.2). Preliminary analysis was done to determine if replicates were statistically similar. If homogeny across replicates was confirmed, replicates were combined into a single dataset. Within each study, replicates were found to be statically similar. Statistical analyses for all studies were run as mixed models with the effect of culture vessel blocked as a random variable, one genotype in each vessel, with four vessels per genotype/treatment combination (Ganju 2000). Genotype was treated differently across studies. In the multiplication rate across 22 genotypes and incubation temperature trials, genotype was treated as the principle independent variable. In the incubation

temperature trial, genotype was also examined for interaction with temperature. For all other trials, genotype was included in the mixed model as a random variable. A Tukey HSD test was used to determine the statistical difference between treatment levels. All statistical tests were run in R (version 3.5.1) with the packages lme4 (version 1.1 - 19), nlme (version 3.1 – 13.7), and multcomp (version 1.4 – 8) (Hothorn et al. 2008; Bates et al. 2015; Pinheiro et al. 2018; R Core Team 2018). The mixed models were assessed with lme4, nlme, and multcomp. The TukeyHSD analysis was conducted with the base R program.

### 3.4. Results

#### *Multiplication rate*

Growth stage of the explant affected the axillary shoot multiplication rates in culture (Fig. 2). The greatest average multiplication rates (7.87 ± 0.38/6 wk) were observed in cultures of seedling-stage explants. The cultures with the least multiplication rates (2.95 ± 0.20/6 wk) were derived from embryos formed from immature zygotic embryos. Explants from mature-stage tissue had an intermediate multiplication rate (5.18 ± 0.31/6 wk).

**Genotype variability in multiplication rate**

Multiplication rates varied considerably between different genotypes, ranging from 1.45 ×/12 wk (Zrite) to 13.25 ×/6 wk (Mettler) (Fig. 3).

**Temperature and isotherm of origin**

Multiplication rate was affected by temperature for three of the eight genotypes tested, and was not related to isotherm of origin (Table 3.3). One genotype (Light Cemetery) had significantly greater height growth and shoot multiplication rate at 21 ± 1 °C, and two genotypes (Mettler and New 11) produced significantly greater height and shoot multiplication at 31 ± 1 °C. Average STN rate across all genotypes in the 31 ± 1 °C treatments was 35.60 ± 5.88 %, while at 21 ± 1 °C it was only 4.27 ± 2.45 %. Genotypes responded similarly to incubation temperature across growth stages.

#### *Incidence of STN*

**BAP**

At higher rates of BAP (2.00 µM and 4.00 µM), rates of STN were reduced relative to the lower BAP treatments (0.49 µM and 0.89 µM) while shoot height and microshoot production rate were unchanged (Table 3.4). STN at 4.00 µM was the least overall at 1.40 ± 0.02 %, compared to the 0.49 µM treatment (17.60 ± 8.06 %).

**Ca, Mg, and B**

Plants exposed to 3.0 mM or 6.0 mM Ca, or combined 6.0 mM Ca and 3.0 mM Mg, generally performed more poorly than the 0.0 mM Ca control, with reduced microshoot production rate and smaller shoots (Table 3.4). Increased Ca (6.0 mM) significantly reduced levels of STN to 12.50 ± 3.76 % compared with the control (20.42 ± 2.85 %). However, multiplication rate for this treatment was also significantly less than the control. When additional Mg was included, STN and growth rates did not differ from the 6.0 mM Ca treatment. At the range of B used, plants did not perform better than the negative B control (Table 3.4). Shoot height was the same across all treatments. The % STN was either the same as the control, or significantly worse.

Similar results occurred in the combined Ca and B trial (Table 3.4). No treatments were significantly different from the control for either multiplication rate or % STN. The 6.0 mM Ca treatment resulted in the lowest growth indicators overall, but did not differ significantly from the control.

**Gelling agent**

STN rate did not differ significantly between treatments or gelling agents (Table 3.4). Multiplication rate differed between the 7.0 g/L and 8.0 g/L agar treatment and all gellan gum treatments (3.51 ± 0.39 – 4.53 ± 0.42 for agar treatments respectively, compared to 6.37 ± 0.44 – 6.66 ± 0.42 across gellan gum treatments). The multiplication rate across all other treatments was statistically similar, ranging from 4.87 ± 0.44 – 6.44 ± 0.55. In the 6.0 g/L agar treatment, similar shoot height, microshoot production rate, multiplication rate, and STN occurred compared with all gellan gum treatments.

## 3.5. Discussion

### *Multiplication rate*

**Growth stage of source material**

Growth stage of the explant had a significant effect on axillary shoot multiplication rate. Explants from juvenile-phase tissue generally respond to culture with greater relative vigor than explants derived from mature-phase tissues (McCown 2000). The outcome of this study with *C. dentata* cultures generally supports these findings, with some differences. Cultures derived from 4 mo old seedlings were the most vigorous overall, with the greatest average multiplication rate (7.87 ± 0.38). However embryo-derived tissues, which can also be classified as juvenile-phase tissue, were the least vigorous, with a multiplication rate of only 2.95 ± 0.20. Genotypes derived

from embryo-stage material were generated via somatic embryogenesis (Merkle et al. 1991). The process of somatic embryogenesis may have left an epigenetic mark on these genotypes, modifying their physiological profile in culture. This phenomenon has been observed during the axillary shoot culture of *Daphne cneorum* (Marks and Myers 1994). Selectively culturing either apically dominant microshoots or microcuttings with a greater proliferation of smaller microshoots in culture resulted in a divergence in phenotype over time, which impacted both rooting and growth regulator response across the two divergent phenotypes. In selecting for an apically dominant shoot, Marks and Myers (1994) may have been indirectly selecting two specific epigenetic profiles of endogenous auxin/cytokinin, one high in cytokinin (apically dominant) and one low in cytokinin (greater shoot proliferation; Su et al. 2011). Somatic embryogenesis requires a narrow concentration of auxin to cytokinin, with auxin generally being required in greater amounts than cytokinin (Corredoira et al. 2015). The process of somatic embryogenesis with *C. dentata* may modify the auxin/cytokinin profile in such a way that cytokinin expression is less than in genotypes derived from seedlings or mature-stage tissues. Alternatively, published protocols for the somatic embryogenesis in *C. dentata* may indirectly select for genotypes with lesser cytokinin expression. Somatic embryogenesis in *C. dentata* has a high failure rate during explant induction (failure of 97.00 – 99.98 %) (Merkle et al. 1991; Johnson et al. 2007). It is possible that only a very narrow allelic profile, low in endogenous cytokinin, can undergo somatic embryogenesis from immature zygotic embryos in *C. dentata*, and this particular profile is recalcitrant to axillary shoot production.

**Temperature and isotherm of origin**

Genotypes included in this study are derived from a similar isotherm (4 – 10 °C), suggesting genotypes should have a similar response to incubation temperature. However, for the majority of eight genotypes examined at two incubation temperatures, there were measurable differences in growth across the two tested temperatures. Three of these genotypes (Mettler, New 11, Light Cemetery) had a difference in multiplication rate between the two temperature treatments of $\geq 2\times$. Two of the genotypes (Mettler, New 11) examined had a greater multiplication rate at 31°C ± 1 temperature than at 21°C ± 1. This is surprising, as 31 °C ± 1 is much greater than the recommended culture incubation temperature for this species (21 – 25 °C) (Key and Cech 1982; Serres et al. 1990; Merkle et al. 1991; Xing et al. 1997; Andrade and Merkle 2005; Yang et al. 2009). Significantly greater STN rates were recorded for all genotypes grown at 31 °C ± 1

compared with 21 °C ± 1. STN incidence was not great enough to reduce multiplication rates below the 21 °C ± 1 treatment for Mettler and New 11. However, these microshoots may be suboptimal for ex vitro transfer and would likely require some adjustment to the protocol before moving microshoot to greenhouse conditions in Stage IV.

Given that three of the eight genotypes produced a higher multiplication rate at one incubation temperature compared with the other, it is surprising that culture temperature is rarely investigated in the literature. Likely, this is an indirect result of the often limited genetic base utilized in tissue culture and the challenges (particularly the costs) of conducting temperature trials. Generally in a study, a single genotype, or a very limited number of genotypes are tested (Xing et al. 1997). Others feature genotypes already commonly used in the literature, which implies that protocols have already been optimized and no further research is necessary. This study highlights the utility of investigating temperature conditions for axillary shoot culture of a species.

**Genotype variability in multiplication rate**

Multiplication rates varied considerably among genotypes, ranging from 1.45 × every 12 wk (Zrite) to 13.25 × multiplication rate every 6 wk (Mettler). In a commercial setting, multiplication for this species would likely be fixed to a subculture cycle of 6 wk only and recalcitrant genotypes requiring 12 wk, such as 664 or Zrite, would likely be dropped unless there was a particular market demand. However, 664 is derived from one of the remaining extant genetically distinct Canadian trees (Husband 2016). There is value to the overall conservation of the species in preserving this recalcitrant genotype, which may very well represent alleles not common in the larger *C. dentata* meta-population. Although this applied to the overall genetic diversity of a species, this principle can also be applied in the preservation of specific select lines. The 664 mother tree in particular was noted for possible blight tolerance by the CCC during the 2014-2015 field survey of Van Drunen et al. 2017 (D. Galic (personal communication, 2017)).

Only 22 genotypes were examined in this study, which for most species would represent too small of a population sample size to assess overall phenotypic diversity in a species. However, extant *C. dentata* populations are at a small fraction of their historic levels, and in many cases continue to decline. Only 1,200 trees are known from the native range of Canada, 80 % of which are < 20 cm in diameter at breast height (DBH) (Van Drunen et al. 2017). The meta-population of *C. dentata* in the United States has not been as extensively surveyed across the entire population. However, genetic diversity in remaining populations is low compared with other *Castanea* spp.

(Huang et al. 1998). The genotypes available for this study were not equally sampled from the remaining *C. dentata* meta-population, and therefore should not be taken as a representative sub-sample of the entire extant *C. dentata* meta-population. However, given how few *C. dentata* remain in the wild, 22 genotypes can be seen as a significant number. For comparison to the Canadian sub-population, 22 genotypes represents 2.0 % of the remaining population (Van Drunen et al. 2017). As well, long term restoration studies have found that a small genetic base can still produce significant increases in genetic diversity over time (Pierson et al. 2007). In a population of *C. dentata* generated by 10 founding plants in the 1880s (9 of which were still alive in 2005), a 2007 study found that the 5,000 descendants were on average, 7.3 % more heterozygous than the founding trees. In context of conservation, there is value in preserving as many genotypes as possible in culture.

***Incidence of STN***

**BAP**

At the greatest concentration of BAP in the medium (4.0 µM), STN was reduced relative to the control, with no difference in shoot height. Work with *C. sativa* suggests that BAP may be the most important component lacking in apical tips and contributing to STN (Piagnani et al. 1996). Directly applying 5 µmol of BAP to the apical shoot tip in *C. sativa* was found to completely eliminate the incidence of STN, compared to an occurrence of 76.7 % in control plants. These results suggest that a lack of endogenous cytokinin is likely contributing to the incidence of STN in *C. dentata*. However, STN rates were reduced but not eliminated at 4.0 µM BAP, unlike results seen in Piagnani et al.'s (1996) study. It remains to be determined if there is a level of exogenous BAP in the medium which can further reduce or eliminate STN in *C. dentata*. Higher rates of cytokinin in the medium may cause a decrease in shoot height, and therefore adding BAP beyond 4.0 µM may negatively impact multiplication rate. As well, one study reported a significant drop in Stage IV rooting rates in *C. sativa* as a result of external application of BAP to the growing shoot tip, an undesirable outcome in a conservation protocol (Vieitez et al. 1989). It is possible that only a properly functioning root system can provide the endogenous cytokinin levels required for the elimination of STN in *C. dentata* (Kurup et al. 2018).

**Ca, Mg, and B**

Imbalances of Ca, Mg, and B in the medium do not seem to be contributing to STN in *C. dentata*. Decreases in shoot growth and multiplication rate resulted from 6.0 mM additional Ca to

the medium. In *C. sativa*, direct application of external calcium (3.0 mM) to apical tips during ex vitro rooting reduced the incidence of STN during Stage IV (to 33.3 % from 76.7 %; Piagnani et al. 1996). With *C. sativa*, even the application to shoot tips of agar with no additional calcium provided enough calcium (at 0.39 ± 0.02 nM) to alleviate STN to some degree (reduced to 30.0 % from 76.7 % for the control). The fact that an external application of calcium can improve STN directly implicates Ca in STN. However, the results seen in this study suggest that increasing Ca in the medium does not alleviate STN in *C. dentata*. Given the results seen in Piagnani et al.'s (1996) study with *C. sativa*, STN in *C. dentata* is likely related to low transpiration rates preventing sufficient Ca translocation to the apical shoot tip (George and de Klerk 2008). The decreased shoot height and microshoot production rate in higher concentrations of added Ca (6.0 mM) also suggest that at these concentrations, added Ca is increasing the osmotic potential of the medium to a degree that it may be further decreasing transpiration rate. Added exogenous Mg (3.0 mM) has been utilized in conjunction with 6.0 mM Ca in published protocols for the axillary shoot culture of *C. dentata* (Xing et al. 1997). However, we did not find that adding 3.0 mM Mg impacted growth or % STN. Alone, B did not improve growth or reduce % STN in *C. dentata*. However, adding B to the 6.0 mM Ca treatment did elevate growth rates to within the range of the control, suggesting that at the concentrations tested in this study, B was interacting with Ca (Abdulnour et al. 2000).

**Gelling agent**

At the gelling agent concentrations tested, no measurable difference in % STN occurred. Osmolality and water potential across the different gelling agents and concentrations included in this study have been measured to be similar, ranging from 230 ± 50 to 240 ± 20 mOsm/kg and -11.3 ± 2.7 to -11.6 ± 1.1 Bar respectively (in full strength MS and 30 g/L sucrose) (Scherer et al. 1988). Therefore, differences in treatments are unlikely to be related to differences in osmotic potential. However, at higher concentrations of agar, cytokinin uptake has been noted to be impaired in a species-specific relationship (Bornman and Vogelman 1984). The 7.0 and 8.0 g/L agar treatments may have been impairing uptake of BAP. However, this would likely have also resulted in an increased % STN. Alternatively, increased concentrations of gelling agent would also have increased concentrations of mineral impurities (Debergh 1983). It is possible that key micronutrients which are available at higher levels in agar relative to gellan gum, such as B, may reach levels toxic to plant growth in *C. dentata* at agar concentrations of 7.0 and 8.0 g/L.

## Conclusions

### *Optimizing a conservation framework at the genotype level*

The results of this study highlight some of the difficulties inherent in applying a tissue culture methodology in a conservation context. Of the 22 genotypes investigated in this study, two required a modification to subculture frequencies, and three (out of eight) showed significant differences in multiplication rate due to incubation temperature. In order to optimally propagate these 22 genotypes in axillary shoot culture, three separate protocols varying in incubation temperature and subculture cycle would be required. Likely as the number of individual genotypes in culture increase, new variation in culture conditions would be discovered and further adjustments would be needed.

However, there are some genotype-independent factors impacting growth in *C. dentata* identified in this study. Growth stage of the source material has a significant impact on relative vigor in culture. Seedling source material can be used to maximize growth rate. However, mature-phase source material can also be propagated effectively, and will prove valuable in conserving existing phenotypically-described mature trees. As well, for the medium components tested, genotypes responded similarly. The incidence of STN was successfully alleviated in this study through elevated exogenous BAP in the medium. However, it remains to be determined if the elevated BAP treatment investigated in this study would have interfered with rooting rates during Stage IV, as was documented with *C. sativa* (Vieitez et al. 1989). Given results observed in *C. sativa*, likely STN in *C. dentata* is also related to issues with translocation, and this cannot readily be alleviated through additional exogenous medium components. This work shows the utility of axillary shoot culture in the short term conservation of *C. dentata*, and provides a framework with which to guide future short term conservation projects with other woody plant species.

## 3.7. Acknowledgements

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**Table 3.1.** Origin of source material for genotypes included in this study.

| Number | Genotype | Year of Explant | Location | Growth Stage of Source Material | Source of Material[†] |
|---|---|---|---|---|---|
| 1 | Alessi | 2011 | Northern NY, USA | Immature zygotic embryos | 1 |
| 2 | Ellis1 | 2006 | Deposit NY, USA | | |
| 3 | Zoar | 2013 | Zoar Valley, NY, USA | | |
| 4 | New 11 | 2015 | Michigan, USA | Seedling (4 mo old) | 2 |
| 5 | New 15 | | | | |
| 6 | New 16 | | | | |
| 7 | New 17 | | | | |
| 8 | New 19 | | | | |
| 9 | AcGraft[*] | 2014 | Nova Scotia, Canada | | 3 |
| 10 | ASH1[*] | | | | |
| 11 | Dundas | 2017 | Southern Ontario, Canada | Mature scion/seedling rootstock | 4 |
| 12 | Hamilton Drive | | | | 5 |
| 13 | IIPP | | | | |
| 14 | LCEM | 2016 | | | |
| 15 | Light Cemetery | 2017 | | | |
| 16 | Mettler | | | | |
| 17 | Siebenmorgen | | | | |
| 18 | Ward | | | | |
| 19 | 664 | | | | |
| 20 | Deacon #2 | 2013 | Buffalo, NY, USA | Stump sprouts (forced indoors) | 1 |
| 21 | Deacon #5 | | | | |
| 22 | Zrite | | Zoar Valley, NY, USA | Crown cutting (forced indoors) | |

* Trees growing in Nova Scotia Canada, likely originated from nuts brought by British Loyalists from the USA after the American Revolution. Therefore, these trees are genetically more closely related to genotypes from the USA than to the Canadian meta-population of *C. dentata*.

† 1: TACF – American Chestnut Research and Restoration Project, SUNY-ESF, Drs. William A. Powell and Charles A. Maynard. 2: TACF – Michigan State University, Dr. Dennis Fulbright. 3: Mr. Leslie Corkum, Nova Scotia and the CCC. 4: CCC – Dr. Dragan Galic. 5: CCC.

**Table 3.2.** Summary of experimental design

| Experiment | Treatment Levels | Dependant Variables Assessed | Media<br>Light (µmol $m^{-2}s^{-1}$)<br>Temperature (°C) | Experimental Design |
|---|---|---|---|---|
| Growth stage of source material | Embryo-stage<br>Seedling-stage<br>Mature-stage | Multiplication rate | CAM<br>60 (16/8 day/night cycle)<br>21 ± 1 | 20 microcuttings per genotype/treatment<br>Microshoots assessed after 6 wk of growth<br>3 reps separated by time |
| Genotype variability in multiplication rate | Genotype | | | |
| Incubation temperature and isotherm of origin | 21 ± 1 °C<br>31 ± 1 °C | Microshoot production rate<br>Height<br>Multiplication rate<br>% STN | CAM<br>60 (16/8 day/night cycle)<br>21 ± 1 or 31 ± 1 | |
| Incidence of STN | BAP (µM):<br>0.00, 0.49, 0.89, 2.00, 4.00 | Microshoot production rate<br>Height<br>% STN | CAM with treatment-specific media modifications<br>60 (16/8 day/night cycle)<br>21 ± 1 | |
| | B (mM):<br>0.0, 0.2, 0.3, 0.4, 0.5 | | | |
| | Ca/Mg (mM):<br>0.0/0.0, 3.0/0.0, 6.0/0.0, 6.0/3.0 | Microshoot production rate<br>Height<br>Multiplication rate<br>% STN | | |
| | Ca/B (mM):<br>0.0/0.0, 0.0/0.025, 3.0/0.0, 6.0/0.0, 3.0/0.025, 6.0/0.025 | | | |
| | Gelling agent (g/L) agar or gellan gum:<br>• Agar:<br>6.0, 7.0, 8.0<br>• Gellan gum<br>3.0, 3.5, 4.0 | | | |

**Table 3.3.** Effect of incubation temperature on mean microshoot height, microshoot production rate, multiplication rate, and STN across genotypes.

| | Mean Microshoot Height (cm) | | Microshoot Production Rate | | Multiplication Rate | | STN (%) | |
|---|---|---|---|---|---|---|---|---|
| Genotype | 31°C ± 1 | 21°C ± 1 | 31°C ± 1 | 21°C ± 1 | 31°C ± 1 | 21°C ± 1 | 31°C ± 1 | 21°C ± 1 |
| Light Cemetery | 0.84 ± 0.20$^{c}$ | 2.29 ± 0.19$^{b}$ | 0.57 ± 0.13$^{c}$ | 1.28 ± 0.12$^{bc}$ | 1.58 ± 0.43$^{c}$ | 4.05 ± 0.42$^{b}$ | 26.85 ± 3.33$^{c}$ | 5.36 ± 0.01$^{a}$ |
| Hamilton Drive | 1.15 ± 0.17$^{c}$ | 1.73 ± 0.18$^{bc}$ | 0.90 ± 0.15$^{c}$ | 0.85 ± 0.12$^{cd}$ | 2.31 ± 0.40$^{c}$ | 2.68 ± 0.40$^{bc}$ | 19.91 ± 5.09$^{c}$ | 3.03 ± 3.03$^{a}$ |
| Dundas | 1.56 ± 0.26$^{c}$ | 2.11 ± 0.15$^{bc}$ | 1.05 ± 0.16$^{c}$ | 0.92 ± 0.09$^{cd}$ | 3.08 ± 0.55$^{c}$ | 3.07 ± 0.31$^{bc}$ | 47.37 ± 9.35$^{bc}$ | 0.00 ± 0.00$^{a}$ |
| Mettler | 7.83 ± 0.65$^{a}$ | 1.92 ± 0.21$^{bc}$ | 2.55 ± 0.22$^{ab}$ | 0.85 ± 0.11$^{cd}$ | 11.91 ± 0.96$^{a}$ | 2.78 ± 0.41$^{bc}$ | 31.93 ± 10.42$^{ab}$ | 4.60 ± 2.31$^{a}$ |
| New 11 | 4.73 ± 0.35$^{b}$ | 1.65 ± 0.12$^{bc}$ | 2.80 ± 0.18$^{ab}$ | 1.25 ± 0.12$^{bc}$ | 8.46 ± 0.60$^{b}$ | 3.50 ± 0.25$^{bc}$ | 23.91 ± 5.02$^{ab}$ | 1.28 ± 1.28$^{a}$ |
| New 15 | 1.90 ± 0.38$^{c}$ | 1.16 ± 0.19$^{c}$ | 1.08 ± 0.20$^{c}$ | 0.62 ± 0.12$^{d}$ | 3.28 ± 0.73$^{c}$ | 2.51 ± 0.41$^{c}$ | 73.88 ± 2.86$^{ab}$ | 12.92 ± 6.47$^{a}$ |
| New 16 | 5.17 ± 0.46$^{b}$ | 6.05 ± 0.37$^{a}$ | 2.10 ± 0.17$^{b}$ | 1.60 ± 0.13$^{ab}$ | 8.88 ± 0.87$^{b}$ | 7.42 ± 0.75$^{a}$ | 27.80 ± 8.53$^{abc}$ | 3.95 ± 2.59$^{a}$ |
| New 17 | 5.12 ± 0.32$^{b}$ | 4.45 ± 0.28$^{a}$ | 3.05 ± 0.20$^{a}$ | 1.90 ± 0.14$^{a}$ | 8.03 ± 0.55$^{b}$ | 6.51 ± 0.53$^{a}$ | 33.91 ± 2.38$^{a}$ | 3.03 ± 3.03$^{a}$ |
| **Mean across genotypes** | 3.27 ± 0.35$^{a}$ | 1.87 ± 0.21$^{b}$ | 1.76 ± 0.18$^{a}$ | 1.16 ± 0.12$^{a}$ | 5.94 ± 0.64$^{a}$ | 4.07 ± 0.44$^{b}$ | 35.60 ± 5.88$^{a}$ | 4.27 ± 2.45$^{b}$ |
| **p value of main effects** | | | | | | | | |
| Temperature | < 0.0001* | | < 0.0001* | | < 0.0001* | | < 0.0001* | |
| Genotype | < 0.0001* | | < 0.0001* | | < 0.0001* | | < 0.0001* | |
| Temp. X Genotype | < 0.0001* | | < 0.0001* | | < 0.0001* | | < 0.0001* | |

Note: Data represents the mean of three replicates ± the standard error. For the means in the same column, letters indicate significant differences (Tukey HSD, $p < 0.05$) within the column. Asterisk indicates significance as determined by mixed model with container as a random variable, where $p < 0.5$.

**Table 3.4.** Effect of treatment levels of BAP, Ca, Mg, B, agar, and gellan gum on the mean microshoot height, microshoot production rate, multiplication rate, and STN in axillary shoot cultures of *C. dentata*.

| Modified Medium Component in CAM | | | | | | Mean Microshoot | | | |
|---|---|---|---|---|---|---|---|---|---|
| BAP (μM) | Ca (mM) | Mg (mM) | B (mM) | Agar (g/L) | Gellan (g/L) | Height (cm) | Microshoot Production Rate | Multiplication Rate | STN (%) |
| 0.00 | | | | | | 0.00 ± 0.00[b] | 0.00 ± 0.00[b] | | 0.00 ± 0.00[b] |
| 0.49 | | | | | | 3.30 ± 0.29[a] | 1.50 ± 0.12[a] | | 17.60 ± 8.06[a] |
| 0.89 | | | | | | 3.30 ± 0.29[a] | 1.38 ± 0.10[a] | | 19.30 ± 5.01[a] |
| 2.00 | | | | | | 3.10 ± 0.28[a] | 1.37 ± 0.11[a] | | 7.30 ± 2.11[ab] |
| 4.00 | | | | | | 2.53 ± 0.28[a] | 1.23 ± 0.11[a] | | 1.40 ± 0.02[b] |
| | 0.00 | 0.00 | | | | 3.46 ± 0.19[a] | 1.78 ± 0.09[a] | 5.13 ± 0.29[a] | 20.42 ± 2.85[a] |
| | 3.00 | 0.00 | | | | 2.79 ± 0.18[b] | 1.42 ± 0.08[b] | 4.43 ± 0.29[ab] | 16.42 ± 0.49[ab] |
| | 6.00 | 0.00 | | | | 2.20 ± 0.16[b] | 1.30 ± 0.07[b] | 3.69 ± 0.26[b] | 12.50 ± 3.76[b] |
| | 6.00 | 3.00 | | | | 2.39 ± 0.19[b] | 1.35 ± 0.08[b] | 3.74 ± 0.29[b] | 17.54 ± 1.76[ab] |
| | | | 0.00 | | | 3.51 ± 0.35[a] | 1.45 ± 0.11[a] | | 8.22 ± 3.03[ab] |
| | | | 0.20 | | | 3.73 ± 0.27[a] | 1.57 ± 0.09[a] | | 11.63 ± 3.93[a] |
| | | | 0.30 | | | 2.74 ± 0.32[a] | 1.18 ± 0.08[a] | | 14.46 ± 2.24[a] |
| | | | 0.40 | | | 3.54 ± 0.29[a] | 1.52 ± 0.11[a] | | 6.63 ± 2.41[ab] |
| | | | 0.50 | | | 3.64 ± 0.52[a] | 1.45 ± 0.10[a] | | 9.65 ± 1.53[ab] |
| | 0.00 | | 0.00 | | | 2.22 ± 0.17[ab] | 1.48 ± 0.10[a] | 4.60 ± 0.38[ab] | 8.44 ± 1.74[a] |
| | 3.00 | | 0.00 | | | 2.01 ± 0.20[ab] | 1.27 ± 0.11[ab] | 4.59 ± 0.42[ab] | 3.55 ± 0.40[a] |
| | 6.00 | | 0.00 | | | 1.50 ± 0.18[b] | 0.96 ± 0.11[b] | 3.36 ± 0.40[b] | 9.20 ± 2.10[a] |
| | 0.00 | | 0.025 | | | 2.29 ± 0.21[a] | 1.39 ± 0.11[a] | 5.19 ± 0.50[a] | 6.16 ± 0.01[a] |
| | 3.00 | | 0.025 | | | 1.72 ± 0.16[ab] | 1.10 ± 0.09[ab] | 3.78 ± 0.37[ab] | 5.13 ± 0.01[a] |
| | 6.00 | | 0.025 | | | 2.07 ± 0.21[ab] | 1.23 ± 0.11[ab] | 4.55 ± 0.45[ab] | 3.27 ± 0.01[a] |
| | | | | | 3.00 | 3.24 ± 0.27[ab] | 1.72 ± 0.12[ab] | 6.37 ± 0.44[a] | 5.83 ± 1.82[a] |
| | | | | | 3.50 | 3.33 ± 0.28[ab] | 1.60 ± 0.13[ab] | 6.44 ± 0.55[a] | 5.68 ± 2.05[a] |
| | | | | | 4.00 | 3.54 ± 0.23[a] | 1.87 ± 0.12[a] | 6.66 ± 0.42[a] | 9.65 ± 4.59[a] |
| | | | | 6.00 | | 2.57 ± 0.24[abc] | 1.48 ± 0.12[abc] | 4.87 ± 0.44[ab] | 4.61 ± 1.86[a] |
| | | | | 7.00 | | 1.74 ± 0.20[c] | 1.16 ± 0.12[c] | 3.51 ± 0.39[b] | 7.04 ± 5.74[a] |
| | | | | 8.00 | | 2.29 ± 0.22[bc] | 1.35 ± 0.12[bc] | 4.53 ± 0.42[b] | 6.17 ± 6.67[a] |
| **p value of main effects** | | | | | | | | | |
| BAP | | | | | | <0.0001* | <0.0001* | | <0.0001* |
| Ca/Mg | | | | | | <0.0001* | <0.0001* | 0.0007* | <0.0094* |
| B | | | | | | 0.1503 | 0.0772 | | 0.0149* |
| Ca/B | | | | | | 0.0231* | 0.0060* | 0.0333* | 0.3957 |
| Gelling agent | | | | | | <0.0001* | 0.0004* | <0.0001* | 0.3114 |

Note: Data represents the mean of three replicates ± the standard error. For the means in each treatment regime, letters indicate significant differences (Tukey HSD, $p < 0.05$) in the same column. Asterisk indicates significance as determined by mixed model with container and genotype as a random variable, where $p < 0.5$.

**Figure 3.1.** STN (large arrow) and expanding lateral bud (small arrow) in microshoot of *C. dentata.*

**Figure 3.2.** Multiplication rate of *C. dentata* axillary shoot cultures across growth stages of source material. Bars represent standard error across three replicates. Different letters indicate that means were significantly different (Tukey HSD, $p < 0.05$).

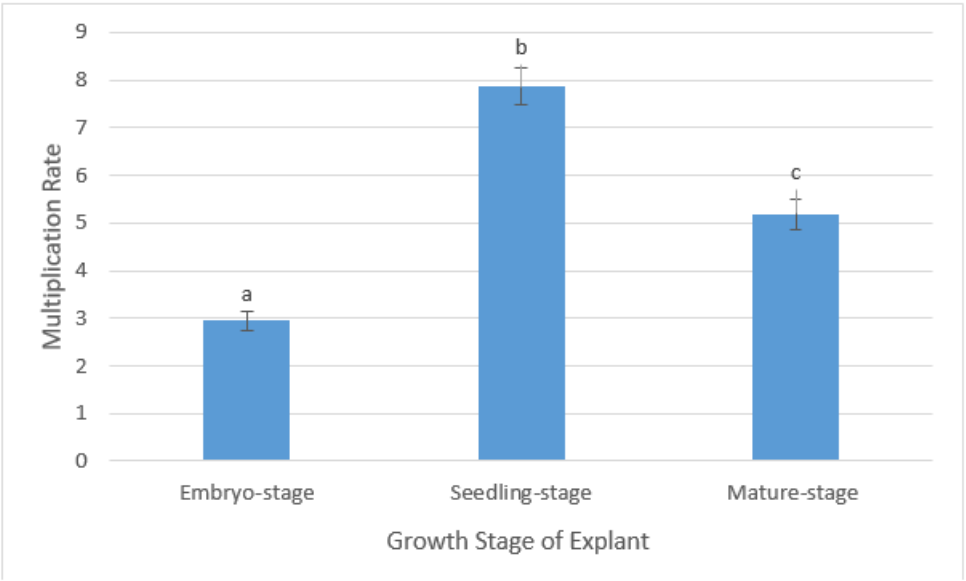
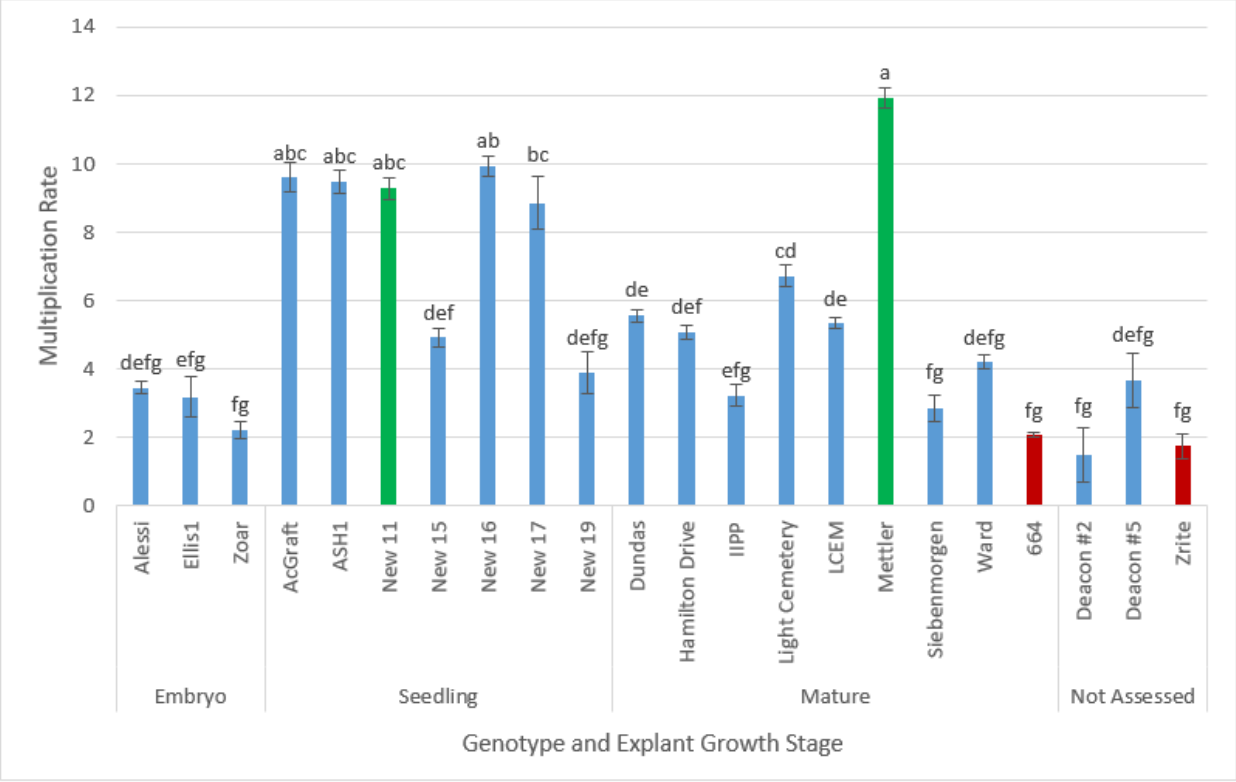


**Figure 3.3.** The range in multiplication rate of all genotypes (over 6 or 12 wk), organized into the three classes for growth stage comparison, and those not assessed. Note that red denotes genotypes where multiplication rate is measured over 12 wk, and green denotes genotypes incubated at 31 ± 1 °C to maximize multiplication rate. All other cultures were incubated over a 6 wk subculture cycle at 21 ± 1 °C. Bars represent standard error across three replicates. Different letters indicate that means were significantly different (Tukey HSD, $p < 0.05$).



## CONNECTING STATEMENT II

In the previous chapter, axillary shoot culture was optimized across 22 genotypes of the declining nut tree species *C. dentata*, in order to build a short term conservation framework for the species. Somatic embryogenesis is also a versatile tool for short term conservation (Corredoira et al. 2017). Somatic embryogenesis can be used to regenerate genetically transformed tissues, may give rise to beneficial somaclonal variants, can be used to increase the genetic variation in low diversity populations, and can be used for high volume propagation purposes when germplasm stability is not a requirement (Del Carmen Vidal and García 2000; Fehér 2015; Ali and Ray 2018; Mao et al. 2018; Żabicki et al. 2018). A protocol for somatic embryogenesis has been developed for *C. dentata* (Merkle et al. 1991). However, this protocol is limited by the existing Stage I. Only immature zygotic embryos harvested 6 – 7 wk post-anthesis have been successfully induced into culture, and success rates for somatic embryogenesis were exceedingly low (0.02 – 3.0 %) (Merkle et al. 1991; Xing et al. 1999; Johnson et al. 2007; Merkle et al. 2011). In the following study, several cutting types from axillary shoot cultures and growth regulator combinations were investigated for the induction of somatic embryos in *C. dentata*. This was done to develop a somatic embryogenesis protocol which would address the most immediate issues with existing protocols, including improving Stage I success rates beyond the reported 0.02% - 3.00 %, and utilizing the more readily available axillary shoot cultures as microcuttings for somatic embryogenesis.

# CHAPTER 4

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## Advances in the indirect organogenesis of American chestnut (*Castanea dentata* (Marsh.) Borkh.) as a short term conservation method

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**4.1. Abstract:**

Current protocols for somatic embryogenesis in the functionally extinct *Castanea dentata* (Marsh.) Borkh. are suboptimal for transformation or conservation purposes. A mere 0.02 - 3.0 % success rate for somatic embryo induction was reported from immature zygotic embryos harvested 6 – 7 wk post-anthesis. The current study involved investigating treatment regimens varying in auxin (NAA, 2,4-D) and cytokinin (BAP, TDZ) content on seven genotypes for induction of somatic embryos. Light regime (16/8 h day/night cycle or 24 h darkness), temperature (21 ± 1 °C or 31 ± 1 °C), and type of microcutting (internodal sections, leaf sections, or single node cuttings) were also compared. From leaf and internodal stem sections, a combination of 10.0 µM NAA and 1.0 µM BAP induced callus that produced branching adventitious roots. A combination of 1.0 µM 2,4-D and 1.0 µM BAP induced callus and somatic embryos on single node cuttings. Somatic embryos developed indirectly, and reached the heart stage for 2 of 3 genotypes. Abiotic conditions were not a focus of this study. However, their possible effect on the successful outcome of this study is discussed in relation to the biology of *C. dentata*. Conservation applications for this work are also discussed.

Keywords: adventitious root formation, light regime, somatic embryogenesis, temperature

## 4.2. Introduction

American chestnut (*Castanea dentata* (Marsh.) Borkh.) is a rare and declining nut tree ranging along the east coast of North America from Southern Ontario (Canada) to central Florida (USA) (Van Drunen et al. 2017). *C. dentata* was formally a keystone primary producer in forest ecosystems, and commercially valued for its high quality lumber, high tannin concentration for tanning hides, and nutritious and plentiful nuts. Since the 1850s, *C. dentata* populations have declined by over 99.9 %. The decline of this species is directly linked to the introduction of the non-native fungal pathogen, *Cryphonectria parasitica* (Murr.) Barr, at the turn of the 20$^{th}$ century (Anagnostakis 2016). In the USA, the majority of extant *C. dentata* stands are highly managed seed orchards. In Canada, remaining trees exist in unmanaged sites, often in mixed forests or urban areas (Van Drunen et al. 2017). Most remaining large individual trees in Canada have been named and phenotypically described. Over 100 yr of breeding work has been invested in the preservation of *C. dentata* as a species (Steiner et al. 2017). However, limitations in described propagation and breeding systems for this species hamper conservation efforts.

*C. dentata* is self-incompatible, necessitating the use of clonal propagation in maintaining desired select genotypes. Traditional clonal propagation techniques (stem cutting and grafting) have very low success rates for *C. dentata* (Galic et al. 2014; Galic et al. 2016). Stem cuttings do not root readily from either mature or juvenile-phase tissue. Adventitious root formation in stem cuttings is inhibited by two endogenous derivatives of ellagic acid (Vieitez et al. 1988). Grafting or stool bed layering are possible, but described procedures have a high failure rate (less than 15.0 % success) or are lengthy to complete (Galic et al. 2014). No mechanism has been proposed for the failure rate of grafts or stool bed layering, although biochemical incompatibilities may be limiting graft success (Santamour 1988). Axillary shoot culture has been utilized on a limited basis for clonal propagation, although issues remain to be resolved with micropropagated shoot quality and ex vitro survival (Xing et al. 1997; Oakes et al. 2016).

As part of the larger conservation and breeding effort of the TACF, a transformation system for *C. dentata* has been developed utilizing somatic embryos (Polin et al. 2006; Andrade et al. 2009). This current methodology is severely limited. The somatic embryo induction rate ranges between 0.02 – 3.00 %, with most genotypes of *C. dentata* that were evaluated recalcitrant to this procedure; significantly limiting the gene pool available for transforming individuals (Merkle et al. 1991; Carraway and Merkle 1997; Xing et al. 1999; Johnson et al. 2007; Andrade et al. 2009;

Merkle et al. 2011). As well, only explants derived from immature zygotic embryos harvested 6 – 7 wk post-anthesis yielded somatic embryos (Merkle et al. 1991). As such, only heterozygous seeds with undefined phenotypes were utilized as source material for breeding, and not a described clone (McCown 2000). In short, the current somatic embryogenesis protocol has limited applicability and utility as a conservation tool for *C. dentata*.

The goal of this study was to develop a method to increase the induction rate of explants for somatic embryogenesis, and to produce somatic embryos from non-zygotic (preferably mature) tissues. To attain this goal, three different empirical strategies were developed from the existing literature on somatic embryogenesis in *C. dentata* and the closely related *C. sativa*. These strategies involved comparisons between basal media, growth regulator ratios, temperature and lighting conditions as well as genotype and microcutting type (Table 4.1). The development of improved methods will greatly facilitate the breeding and conservation work needed for *C. dentata*.

## 4.3. Materials and methods

### *Source material*

Genotypes were maintained as axillary shoot cultures before microcuttings were harvested for this study. Genotypes used in this study were derived from different source populations within the larger meta-population, and from material of varying growth stages. Seeds of open pollinated American chestnut stands in Michigan were provided by the Michigan Chapter of the TACF, and vernalized (4 mo cold-moist stratification in peat moss at 4 °C) before planting into a seedling growth chamber maintained at 21 ± 1 °C at 100 µmol $m^{-2}s^{-1}$ of cool white fluorescent light on a 16/8 h day/night cycle. Genotypes New 11, New 16, and New 17 were derived from explants removed from 4 mo old seedlings. The New York State Chapter of the TACF provided axillary cultures of Ellis1, which was originally derived from an immature zygotic embryo at approx. 6 - 7 wk post fertilization (Merkle et al. 1991). Seeds of open pollinated genotypes, including trees from seeds brought to Nova Scotia by British Loyalists in the late 1700s were provided by Mr. Leslie Corkum of the CCC. These seeds were vernalized, germinated, and maintained in identical conditions to the seeds obtained from Michigan. From these, genotypes AcGraft and ASH1 were derived from explants taken from 4 mo old seedlings. The CCC provided grafts of mature-phase scion tissues of select named Canadian genotypes of *C. dentata* grafted onto open pollinated seedling rootstocks. Genotypes of the Light Cemetery tree and the Mettler tree were derived from mature-phase stem scion tissue on a seedling graft, and have been genotyped to confirm pure *C.*

*dentata* status (Galic et al. 2014). Mettler has proven to be particularly vigorous in culture (see Chapter 3).

Axillary shoot cultures were initiated and maintained on CAM, which consisted of WPM basal salt medium (Lloyd and McCown 1980) with NN vitamins (Nitsch and Nitsch 1969), 30.0 g/L sucrose, 1.0 g/L casein hydrolysate, 6.5 g/L agar (agar; *Gelidium* sp., PhytoTechnology Laboratories), and 1.0 µM BAP, adjusted (prior to autoclaving) to pH 5.6. This medium was extensively modified from Xing et al. (1997) based on results obtained in Chapter II, and preliminary work. Modifications included novel gelling agent and concentration, an increase in BAP concentration and final pH (from 4.5), and the elimination of additional Ca, Mg, IBA, and sequestrene 330 Fe. The number of different basal media recipes for axillary shoot culture was also reduced from three (a different recipe for Stage I, II, and III), to a single medium for Stage I and II. Light level was also increased from the recommended 40 µmol $m^{-2}s^{-1}$.

Axillary shoot cultures for this study were incubated as 5 microcuttings per Magenta© GA7 vessel (80 ml of medium per container) at 21 ± 1 °C, under 60 µmol $m^{-2}s^{-1}$ of cool white fluorescent light on a 16/8 h day/night cycle. All medium components, excluding sucrose, were obtained from PhytoTechnology Laboratories (Lenexa, Kansas, USA). Sucrose (Redpath granulated sugar) was obtained from a local grocery.

***Somatic embryogenesis***

**Strategy 1. NAA/BAP**

Treatment levels were based on studies of somatic embryogenesis in non-zygotic tissues of the closely related *C. sativa* (Corredoira et al. 2003). Medium consisted of WPM with NN vitamins, 0.5 g/L casein hydrolysate, 7.0 g/L agar, 30.0 g/L sucrose, adjusted to pH 5.6. Cultures were incubated at 21 ± 1 °C under cool white fluorescent light (30 µmol $m^{-2}$ $s^{-1}$) for 16/8 h day/night cycle or in 24 h darkness. Eight different growth regulator treatment ratios were tested, including (NAA/BAP µM): 0.0/0.0 (control), 5.4/4.4, 10.8/8.9, 21.5/2.2, 43.0/4.4, 2.0/1.0, 5.0/1.0, and 10.0/1.0. Cultures were incubated in petri dishes (100 x 15 mm) with 30 mL of medium/dish at 21 ± 1 °C under 30 µmol $m^{-2}s^{-1}$ on a 16/8 h day/night cycle or under 24 h darkness.

Microcuttings derived from axillary shoot cultures consisted of internodal stem sections 0.5 cm ± 0.1 cm long or leaf squares 0.5 cm ± 0.1 cm wide centered on the midvein from axillary shoot cultures of AcGraft, ASH1, Light Cemetery, New 16, and New 17. Three internodal stem sections and three leaf cuttings were placed into each petri dish. Per treatment and genotype

combination, 3 petri dish replicates were used for a total of 18 microcuttings. Three replicates were conducted over a period of 7 mo. To prevent tissue necrosis from excessive phenolic oxidation, 24 h after culture initiation, microcuttings were moved to fresh sections of the medium within each dish (Preece and Compton 1991). Over a period of 8 wk, dishes were checked weekly for callus growth and root development.

**Strategy 2 - TDZ**

Medium contained WPM, NN vitamins, 1.0 g/L casein hydrolysate, 6.5 g/L agar, and 30.0 g/L sucrose, adjusted to pH 5.6. A TDZ factorial design derived from Huetteman and Peerce (1993) was used to evaluate seven TDZ treatment levels (µM): 0.0, 1.0, 10.0, 100.0 and (nM): 1.0, 10.0, and 100.0. Source material consisted of genotypes ASH1, Ellis1, and New 11 with microcuttings as described for the NAA/BAP ratio design. Cultures were incubated in petri dishes with 30 ml of medium at 21 ± 1 °C under 30 µmol $m^{-2}s^{-1}$ on a 16/8 h day/night cycle or under 24 h darkness.

Per treatment and genotype combination, 2 petri dish replicates were used, for a total 12 microcuttings. Three replicates were conducted over time, over a period of 5 mo. Cultures were checked weekly over a 6 wk period for callus growth and indirect organogenesis.

**Strategy 3 - 2,4-D/BAP**

This trial was originally designed as a preliminary study for increasing shoot proliferation during axillary shoot culture. Genotypes examined in this study included AcGraft, ASH1 and Mettler. Two separate growth regulator concentrations on the same basal medium, and three separate microcutting treatments were directly compared. A control consisting of the CAM was compared against a CAM with added 1.0 µM 2,4-D.

Microcuttings consisted of either the previously described internodal stem segments and leaf cuttings, or single node stem cuttings. Internodal stem segments and leaf cuttings were cultured in 30.0 mL of medium on petri dishes. Per petri dish, six microcuttings (three leaf cuttings and three internodal stem cuttings) were cultured as previously described. Three petri dishes were used per genotype. Single node cuttings consisted of a single unexpanded axillary bud, a subtending leaf, and a section of stem 0.5 cm ± 0.2 cm in length. Single node stem cuttings were cultured in Magenta© GA7 containers on 80.0 mL of medium, five per container. Four GA7 vessels with a total of 20 single node stem cuttings were examined per treatment. All cultures were placed in a walk in growth room at 31 ± 0.5 °C, at 60 µmol $m^{-2}s^{-1}$, with 24 h light. Trials were

repeated for two replicates over time, over a 1 yr period. Magenta© and petri dish trials were not conducted simultaneously.

*Statistical design*

In all experiments, treatment containers were organized in a completely randomized design (CRD; Compton et al. 2000). Experiments were replicated two or three times. Replications were blocked in time. Comparison of treatment, replicate, and genotype was made using logistic regression. Callus occurrence data was classified categorically and converted into a number system for analysis (Table 4.2). For comparing callus quality in all trials, callus was qualitatively assigned a class from 0 to 4 based on appearance. For the occurrence of adventitious roots or somatic embryos, a binary dataset was generated for the NAA/BAP and 2,4-D/BAP trials. Wilson's score interval was used to calculate the binomial proportion confidence interval (Newcombe 1998). Statistical analysis was conducted with the software R (version 3.5.1) with the package binom (version 1.1-1; Sundar 2014; R Core Team 2018).

## 4.4. Results

***NAA/BAP design***

Callus formed under light was qualitatively different from callus formed in darkness. When callus formed during incubation in light, it was green and nodular (Class 1; Table 4.2). Callus which formed in darkness was either creamy and nodular (Class 2), or white and friable (Class 3). All treatments, except for the control, produced callus. All genotypes generated similar amounts of callus. None of the treatment ratios of NAA/BAP produced somatic embryos. However, two treatment levels did produce prolific branched root systems (Figure 4.1). The formation of adventitious roots began at 3 wk in culture, and occurred at (NAA/BAP μM) 5.0/1.0 and 10.0/1.0 (Figure 4.2). Root formation was rare at 5.0/1.0, ranging from 1 - 3 out of 24 microcuttings, and took place in New 16 only. At treatment 10.0/1.0, all genotypes except for New 17 produced adventitious roots in the majority of microcuttings. Root formation occurred in 11 – 23 microcuttings (out of all three replicates), with New 16 producing by far the greatest number of adventitious roots. No root formation occurred in New 17 under any treatment. Root formation occurred only in the dark, and was of similar quality and quantity when derived from either leaf sections or internodal stem sections. Root quality varied among genotypes. The majority of genotypes developed similar branched root systems with fine root hairs. However, Light Cemetery formed root segments which were short with limited branching.

***TDZ design***

Microcuttings rapidly senesced at (TDZ nM) 0.0, 1.0, and 100.0. At 10.0 µM TDZ, callus occurred only in New 11. The most callus across all genotypes occurred in the 100.0 µM and 1.0 nM TDZ treatments. At 10.0 mM TDZ, callus occurred on Ellis1 and ASH1 but not in New 11 microcuttings. Genotypes differed consistently in callus quality and quantity (Figure 4.3). Ellis1 produced the most callus and callogenesis occurred at the greatest range of TDZ treatment levels. Ellis1 callus was generally cream colored and nodular (Class 2). ASH1 callus was least overall and mainly green and nodular in the light and did not grow in the dark (Class 1). New 11 produced consistent, but low levels, of cream colored callus. Although prolific callus was produced at some TDZ treatment levels across all genotypes, no adventitious organs were produced.

***2,4-D/BAP design***

Somatic embryogenesis was induced by (2,4-D/BAP µM) 1.0/1.0. Microcutting type and genotype affected the formation of somatic embryos. Internodal stem and leaf sections did not callus and deteriorated (Class 1). Single node cuttings produced callus and somatic embryos. Somatic embryos grew mainly from the basal end of the stem or the midvein of the leaf. Where the subtending leaf touched the medium surface, embryogenic callus developed.

The response of Mettler was different from the other genotypes tested. Single node cuttings of Mettler produced less callus overall, did not produce embryogenic callus, and this was the only genotype in which axillary buds underwent bud break and new shoot growth. In contrast, AcGraft and ASH1 produced the greatest proportion of embryogenic callus, and bud expansion and bud break did not occur. Across all replicates, the formation of somatic embryos ranged from 14 – 19 (out of 20) microcuttings in AcGraft and ASH1. Embryogenic callus formation was evident after 4 wk treatment. By 6 wk, globular somatic embryos were observed. By 8 wk, embryos had begun to enter the heart stage, and in some cases the torpedo stage of development (Figure 4.4). Unfortunately by 10 wk, embryos had begun to callus or senesce.

**4.5. Discussion**

***NAA/BAP design***

This study provides evidence that *C. dentata* can be induced to reliably produce adventitious roots in culture. Similar callus quality and root formation across the majority of genotypes examined in this study suggest that different genotypes of *C. dentata* are likely to

respond similarly to additional (NAA/BAP µM) 10.0/1.0. As well, the adventitious roots resemble typical roots formed in field plants, with significant branching and prolific root hairs.

The formation of adventitious roots was not a primary goal of this study. However, adventitious root production following axillary shoot culture of *C. dentata* has proven to be challenging (Corredoira et al. 2017). Microcuttings produce a large subtending vascularized callus during Stage II. A separate rooting stage is necessary for survival (Oakes et al. 2013; Oakes et al. 2016). However, losses can be significant at this stage. If it were possible to produce the same quality and quantity of adventitious roots following axillary shoot culture of *C. dentata*, likely successful ex vitro transplant rates would be increased.

***TDZ design***

In some woody plant species known to be recalcitrant to somatic embryogenesis, TDZ has induced embryogenesis (Bates et al. 1992). The results from this study suggest that TDZ alone, across a wide range of concentrations, does not facilitate somatic embryo production in *C. dentata*.

***2,4-D/BAP design***

A high auxin to cytokinin ratio is generally required for the formation of indirect somatic embryos (Sanglard et al. 2018). This pattern can be seen in the existing protocols for development of somatic embryos from immature zygotic embryos in *C. dentata*. The first successful culture of somatic embryos from immature zygotic embryos occurred at a ratio of (2,4-D/BAP µM) 18.2/1.1 (Merkle et al. 1991). To generate somatic embryos from existing proembryogenic callus, an elevated auxin to cytokinin ratio of (2,4-D/BAP µM) 1.8 /1.1 is currently used (Polin et al. 2006). Somatic embryos have been generated from non-zygotic tissues in *C. sativa* (Corredoira et al. 2003). However, this procedure also involved elevated auxin to cytokinin ratios of (NAA/BAP µM) 5.4/4.4 or 21.5/2.2, which resulted in a low induction frequency (1.0 %). These treatments were used in the NAA/BAP design of this study, but only resulted in callus and adventitious root production.

The results obtained in this study suggest that the production of somatic embryos from non-zygotic tissues in *C. dentata* requires equal concentrations of 2,4-D and BAP for embryo formation. However, growth regulator concentration is likely only one of the factors necessary for somatic embryogenesis. A major factor identified in this study was effect of microcutting type on the development of somatic embryos. Somatic embryos formed only on single node cuttings, not internodal segments or leaf sections. Embryogenic callus ultimately grew on all single node stem

tissues, including the leaf blade and stem portion. Likely the endogenous processes inherent in a larger cutting containing multiple interconnected organs may be providing the correct endogenous hormone balance in concert with the exogenous 2,4-D and BAP in the medium.

Mettler was the only genotype derived from mature-phase source material in the 2,4-D/BAP trial, and did not produce any embryogenic tissues. This should not be taken as an indication that genotypes derived from mature-phase genotypes are not capable of producing somatic embryos in culture (Holtz et al. 2017). The Mettler genotype has an atypical growth form in axillary shoot culture, relative to all other genotypes tested (Chapter 3). Mettler was the most prolific of all genotypes, and consistently produced the largest lateral shoots and grew more quickly than other genotypes (bud expansion began at 1 wk, compared to 2 – 3 wk for other genotypes. Given the extreme differences in relative vigor, it is likely that Mettler has a different endogenous hormone profile than ASH1 or AcGraft, both of which have similar rates of relative vigor in axillary shoot culture (Ezura and Harberd 1995). These observations should also be taken into consideration when attempting to enter genotypes of *C. dentata* into protocols for somatic embryogenesis. The auxin/cytokinin ratio of the medium may need to be adjusted based on the relative growth rate of the genotype being induced. It should not be assumed that a genotype is recalcitrant to somatic embryogenesis before an exploratory trial involving differing concentrations of 2,4-D and BAP.

Although not directly investigated in this study, abiotic factors utilized in this design may have played an important role and merit comment. The successful cultures seen in this study were incubated at an unusually high light level, and were under continuous illumination with cool white fluorescent light. Many studies in somatic embryogenesis consider darkness to be essential to embryo formation. However, some have noted that light incubation, or alternating time periods of incubation in light and darkness, are beneficial to embryo formation (Gray et al. 1993). Light levels during culture incubation are not considered to be a factor influencing somatic embryo formation in *Castanea* (Corredoira et al. 2016). Similarly, in many studies involving somatic embryogenesis in *Castanea*, darkness is considered to be essential to somatic embryo formation (Merkle et al. 1991; Carraway and Merkle 1997; Andrade and Merkle 2005; Polin et al. 2006). In others, light level is considered to be so unimportant that it is not even noted (Merkle et al. 1991; Ballester et al. 2001). No study of *C. dentata* somatic embryogenesis has examined the effect of light level, or made a comparison of light to dark directly. However, *C. dentata* has been documented as having

a high level of light-induced plasticity in the field (Joesting et al. 2009). Individuals in the understory maintain a slow growth rate in the shade, but respond opportunistically to increased light with a drastic increase in growth rate (McEwan et al. 2006). Given the natural light-induced plasticity observed in vivo for *C. dentata*, it may be that light levels play an important role in the development of somatic embryos from non-zygotic tissues. Cultures were also incubated at a temperature much warmer than conventionally used for either somatic embryogenesis in *Castanea*, or axillary shoot culture (García-Mendiguren et al. 2016). Most *Castanea* cultures have been incubated at between 21 °C and 25 °C (Carraway and Merkle 1997; Andrade and Merkle 2005; Polin et al. 2006), while the cultures in this study were incubated at 31 ± 1 °C. Although rarely studied, there is some evidence that in vitro plants respond to external temperatures in culture independently of their preferred in vivo temperature range (García-Mendiguren et al. 2016). Kvaalen and Johnsen (2007) found with *Picea abies* that the greatest number of mature somatic embryos were produced at 28 °C, instead of the culture standard of 23 °C or a lower temperature of 18 °C. They also noted that several of the regenerated plantlets had temperature optima for growth either below 18 °C or above 28 °C, well outside of the usual temperature optima for growth of the source plants (24.4 - 25.6 °C). The authors did not determine if this change in temperature optima was a result of random mutations during somatic embryogenesis, or a temporary plastic response to the tissue culture environment. Similar warm temperatures (28 °C relative to 23 or 18 °C) were found to significantly reduce somatic embryo occurrence in *Pinus radiata* (García-Mendiguren et al. 2016). Therefore temperature response is likely species-specific, and may even be specific to a particular genotype (Chapter 3). There is also evidence that at warmer temperatures in vitro (above 26 °C), plants may respond to increased light more vigorously than if they were grown at ≤ 25 °C (de Capite 1955). Therefore increased temperature may be working synergistically with increased light level. Much more work needs to be done to determine the factors and mechanisms involved in the effect of these abiotic factors on somatic embryogenesis in *C. dentata*. However, the importance of these effects should not be underestimated.

**4.6. Conclusions**

*C. dentata* formed adventitious roots and somatic embryos from non-zygotic tissues in some treatments. It may be possible to utilize the observations here on adventitious rooting to increase rooting rates following axillary shoot culture, and improve transplant survival rates during Stage IV. The production of somatic embryos from axillary shoot culture tissues was shown to be

possible, and to have a much greater induction rate than existing methodologies utilizing immature zygotic embryos as source material. Further work needs to be undertaken to determine if somatic embryos produced from single node cuttings will respond to existing procedures for the growth and germination of *C. dentata* somatic embryos derived from zygotic tissues. It also remains to be determined if mature-phase source plants can be induced to form somatic embryos in culture, as Mettler was not representative of genotypes derived from mature-phase source material. However, if these issues can be resolved, somatic embryogenesis will have increased value for the rapid breeding and propagation of *C. dentata*.

**4.7. Acknowledgements**

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**Table 4.1.** Summary of treatment strategies.

| Strategy | Growth Regulator | Other Medium Components (g/L) | Light (µmol $m^{-2}s^{-1}$) Temperature (°C) | Microcutting | Container | Experimental Design |
|---|---|---|---|---|---|---|
| NAA/BAP | (µM/L)<br>0.0/0.0<br>5.4/4.4<br>10.8/8.9<br>21.5/2.2<br>43.0/4.4<br>2.0/1.0<br>5.0/1.0<br>10.0/1.0 | WPM<br>NN vitamins<br>0.5 casein hydrolysate<br>7.0 agar<br>30.0 sucrose<br>pH 5.6 | 30 (16/8 day/night cycle)<br>24 h darkness<br>21 ± 1 | Internodal stem sections<br>Leaf squares | Petri dish | 18 microcuttings per genotype/treatment<br>3 reps completed over 7 mo |
| TDZ | (µM/L)<br>0.0<br>1.0<br>10.0<br>100.0<br>(nM/L)<br>1.0<br>10.0<br>100.0 | WPM<br>NN vitamins<br>1.0 casein hydrolysate<br>6.5 agar<br>30.0 sucrose<br>pH 5.6 | 30 (16/8 day/night cycle)<br>24 h darkness<br>21 ± 1 | Internodal stem sections<br>Leaf squares | Petri dish | 12 microcuttings per genotype/treatment<br>3 reps completed over 5 mo |
| 2,4-D/BAP | (µM/L)<br>0.0/1.0<br>1.0/1.0 | WPM<br>NN vitamins<br>1.0 casein hydrolysate<br>6.5 agar<br>30.0 sucrose<br>pH 5.6 | 60 (24/0 day/night cycle)<br>31 ± 1 | Internodal stem sections<br>Leaf squares<br>Single node stem cuttings | Petri dish<br>Magenta ©<br>GA7 | 18 microcuttings per genotype/treatment for internodal stem cuttings and leaf cuttings<br>20 microcuttings per genotypes/treatment for single node cuttings<br>For both: 2 reps over 1 yr |

**Table 4.2.** Description of callus classes used to characterize results (magnification 120x).

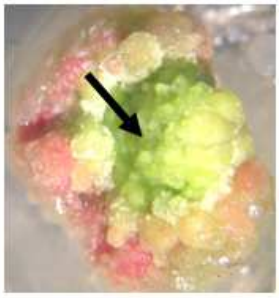
| Class 0 | Class 1 | Class 2 | Class 3 |
|---|---|---|---|
|  |  |  |  |
| No callus, tissue necrosis | Green nodular callus | Creamy nodular callus | White friable callus |

**Figure 4.1.** Callus and adventitious root formation (A) roots emerging from callus growing on a leaf microcutting (B) branching on roots (C) formation of root hairs (magnification A: 120 X, B: 240 X, C: 500 X).

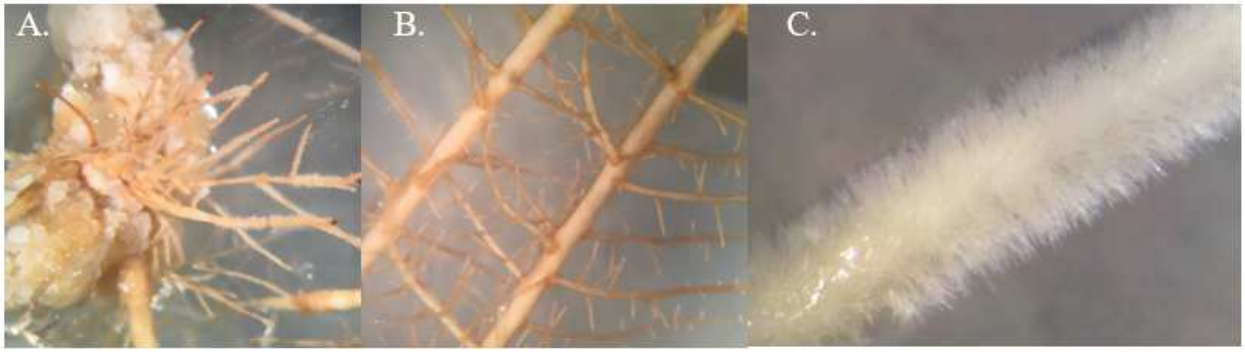



**Figure 4.2.** Greater incidence of adventitious root formation in New 16 (N16) relative to AcGraft (AcG), ASH1, Light Cemetery (LC), and New 17 (N17). Bars represent the binomial proportion confidence interval.

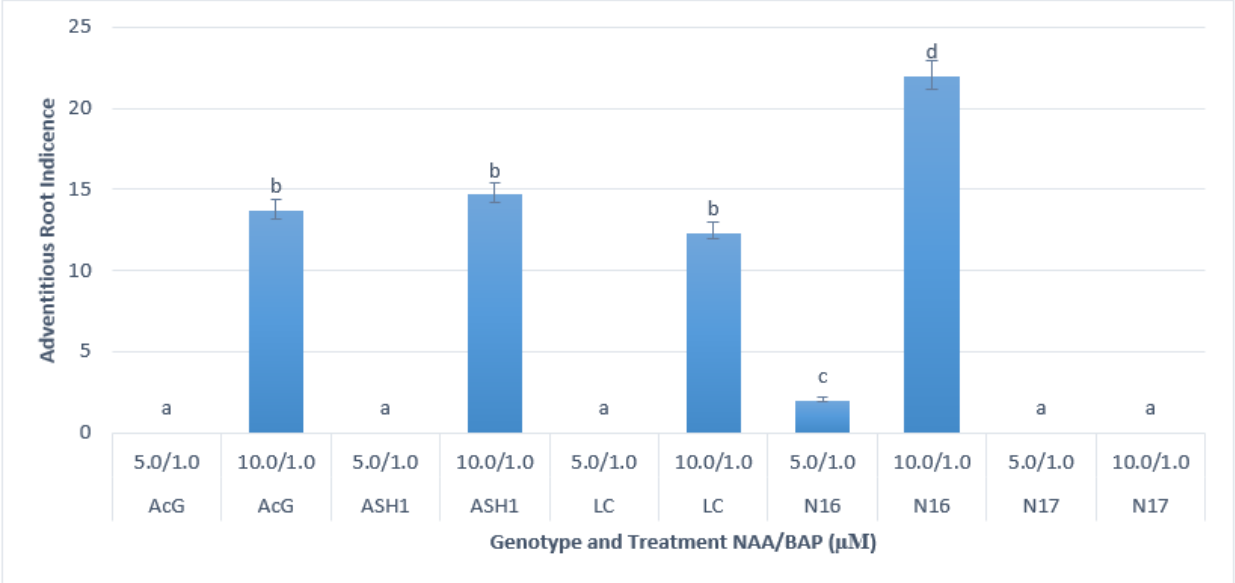
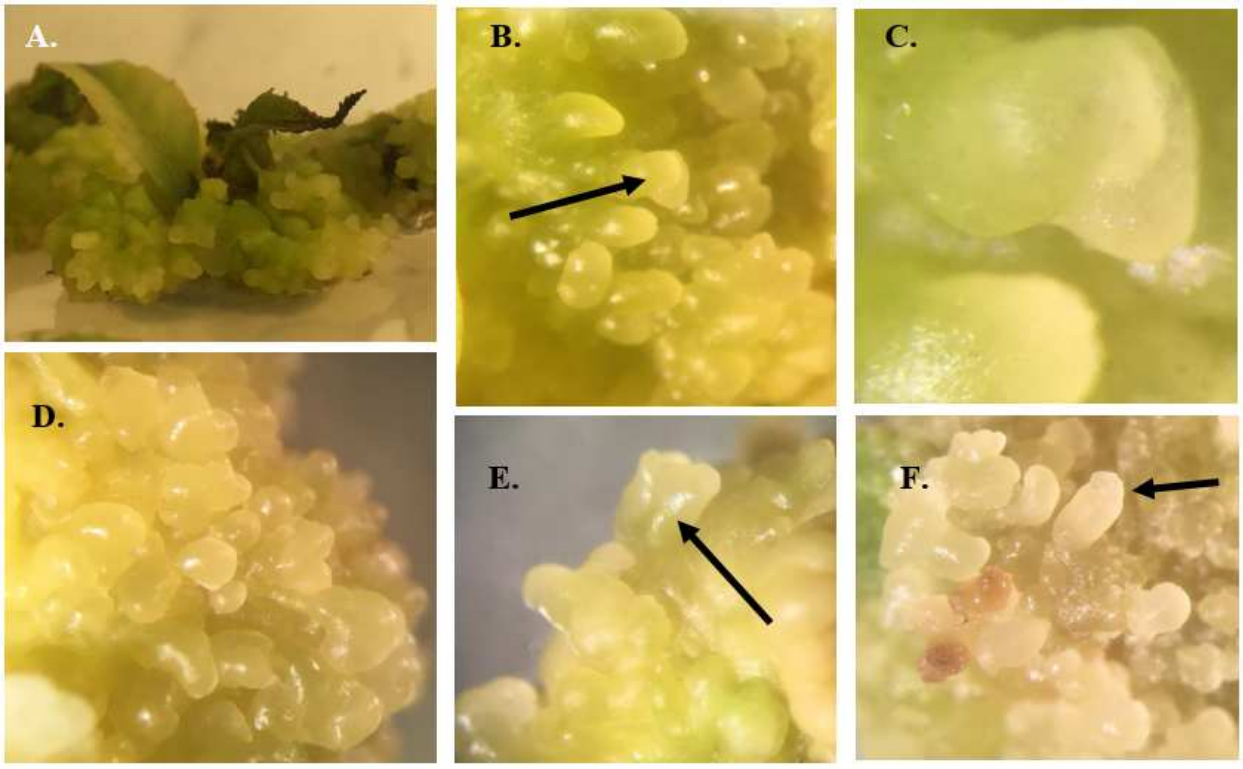


**Figure 4.3.** Callus quality observed in NAA/BAP and TDZ trials (A) green nodular callus (B) creamy nodular callus (C) white friable callus (arrow; magnification 240 X).


**Figure 4.4.** Callus and somatic embryo formation on single node cuttings (A) somatic embryogenesis culture at 8 wk (B-F) globular to early torpedo stage somatic embryos at 6 wk. Heart-shaped embryo highlighted in B can be seen magnified in C. (D) example of high density embryo formation evident on majority of microcuttings. (E) torpedo stage embryo (arrow). (F) elongated embryos (arrow; B, D, E, F: magnification 240 X, C: 500 X).



## CONNECTING STATEMENT III

Chestnut blight is a commercially and environmentally relevant disease of *Castanea* species. The disease affects commercial production in *C. mollissima* and *C. crenata*, nearly destroyed the commercial capacity of *C. sativa*, and has driven *C. dentata* and *C. pumila* to functional extinction in North America (Hepting 1974; Roane et al. 1986; Zhou et al. 1993; Robin and Heiniger 2001; Fu and Dane 2003; Van Drunen et al. 2017). The causal agent of chestnut blight, *C. parasitica*, has been known since 1904 (Merkel 1905). However, despite the importance of this disease internationally, and the length of time in which the causal agent has been known, very little is understood concerning the host-pathogen interaction between *C. parasitica* and *Castanea*.

The development of an efficient system for generating somatic embryos is a useful tool in any transformation system (Merkle et al. 2011; Merkle et al. 2017). The previous study (Chapter 4) has improved upon existing somatic embryogenesis methods in *C. dentata*, with the hopes of improving the utility of somatic embryogenesis as a conservation tool for *C. dentata*. Existing protocols for somatic embryogenesis of *C. dentata* are currently used to transform *C. dentata* in attempts to improve host tolerance to the blight (Polin et al. 2006; Zhang et al. 2011; Zhang et al. 2013). Zhang et al. (2013) have had success in increasing host tolerance through targeting oxalate during blight infection through the expression of oxalate oxidase in *C. dentata* with the insertion of such an oxalate oxidase gene from wheat. Single-gene host defence is frequently overcome by plant pathogens (McDonald and Linde 2002; Sprague 2006). Host resistance can be improved through the use of stacked genes for resistance, a technique which is becoming simpler with the advent of rapid gene cloning and improved transformation such as CRISPR/Cas9 (McDonald and Linde 2002; Barakate and Stephens 2016; Steuernagel et al. 2016). However, for any improvement to host tolerance to be made, mechanisms of the host-pathogen interaction need to be known. To date, very little is known concerning how *C. parasitica* attacks *C. dentata*, and how *C. dentata* defends itself from the pathogen. In the following review, the mechanisms and metabolomics of the interaction between all *Castanea* species and *C. parasitica* are noted. This is the first review of this kind, and details over 150 yr of work. It is the intent of this review to provide a framework for researchers to pursue blight tolerance in *C. dentata* more effectively in the future.

# CHAPTER 5

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## Mechanisms and metabolomics of the host-pathogen interactions between Chestnut (*Castanea* species) and Chestnut blight (*Cryphonectria parasitica*)

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**5.1. Abstract**

Chestnut blight is a stem girdling disease of *Castanea* caused by the fungal pathogen *Cryphonectria parasitica* (Murr.) Barr. Chestnut blight affects all *Castanea* species to some degree. In Asian species, chestnut blight is a commercially relevant disease which primarily affects nut production. In American and European species, chestnut blight has caused significant declines in wild populations, and continues to negatively affect nut production in the European chestnut (*C. sativa*). Despite the profound effect of this disease in the *Castanea* genus, very little is known concerning the factors involved in the host-pathogen interaction between *C. parasitica* and its *Castanea* hosts. This review summarizes information on known mechanisms and metabolites involved in the host-pathogen interaction and contributes original information on the pathogen in relation to susceptible and putatively resistant genotypes with a view to furthering research that will promote a better understanding of this devastating disease and enable its control.

## 5.2. Introduction

The pathogenic fungus *Cryphonectria parasitica* causes a disease within the chestnut genus (*Castanea*) known as chestnut blight (Milgroom et al. 1992; Milgroom et al. 1996; Yan et al. 2007; Mellano et al. 2012). *C. parasitica* is native to China and Japan, and was unintentionally introduced to Europe and North America at the turn of the 20$^{th}$ century. Susceptibility to this pathogen varies widely among *Castanea* species (Table 5.1) (Graves 1950; Dane et al. 2003; Mellano et al. 2012). Asian species (*C. crenata*, *C. henryi*, *C. mollissima*, and *C. seguinii*) are generally tolerant to *C. parasitica*, possibly as a result of a shared evolutionary history. Tolerant species infected with *C. parasitica* generally develop superficial surface lesions or cankers on the trunk, and survive infection with minimal internal damage. In contrast, European (*C. sativa*) and North American (*C. dentata*, *C. ozarkensis*, and *C. pumila)* species are highly susceptible to the pathogen, and infection results in host mortality. In susceptible species, disease symptoms progress rapidly from initial infection to large sunken or swollen stem cankers that lead to complete girdling of the stem.

*C. parasitica* has a significant effect on both commercial and natural populations of *Castanea* worldwide. Within its native range, it has long been believed that *C. parasitica* was an incidental pathogen of Asian native trees (Yan et al. 2007). However, recent surveys and changes in cultural practices (from diverse heterocultures to minimal genotype diversity) suggest that chestnut blight may be increasingly problematic for commercial plantings of *C. mollissima* (Zhou et al. 1993). Outside of its native range, *C. parasitica* was first documented infecting non-Asian *Castanea* species in 1904, when several *C. dentata* individuals in the New York Zoological Garden were observed with rapid crown die-back (Merkel 1905; Hepting 1974). Genetic analyses of North American *C. parasitica* populations suggest that successive waves of introductions probably took place in North America, carried by Japanese trees widely imported through mail order catalogues (Powell 1900; Anagnostakis 1987; Milgroom et al. 1996; Dutesh et al. 2012). North American *Castanea* species are generally highly susceptible to C. *parasitica*. Populations of *C. dentata, C. ozarkensis*, and *C. pumila* have been decimated by chestnut blight (Hepting 1974; Roane et al. 1986). *C. dentata* populations were reduced by over 99.9%. In a little under 40 yr, an estimated 4 billion trees were reduced to a few thousand mature individuals and numerous repeatedly sprouting understory shrubs (Paillet 1984). Populations of *C. ozarkensis* and *C. pumila* were not well studied before the introduction of the blight, but it is commonly believed that these species suffered a similar decline to *C. dentata* (Fu and Dane 2003). Today, both North American species exist

mainly as resprouting stumps, highly managed plantations, and isolated mature trees (Brewer 1995; Van Drunen et al. 2017). In Canada, recent population surveys suggest that the remaining Canadian meta-population of *C. dentata* will face extinction within the next 100 yr (Boland et al. 2012; Van Drunen et al. 2017). In Europe, introduced populations of *C. parasitica* were discovered in 1938 and subsequently ravaged populations of *C. sativa*.

Recent comparisons of microsatellite markers between globally distributed sub-populations of *C. parasitica* suggest that *C. parasitica* was probably introduced to Europe from North America (Dutesh et al. 2012). The application of a naturally-occurring European dsRNA hypovirus resulted in widespread reduction in *C. parasitica* virulence across the continent (Biraghi 1953; Grente 1965; Anagnostakis 1982). Today, chestnut blight is largely controlled in Europe through the hypovirulence mechanism, and the European chestnut continues to be a commercially relevant species (Robin and Heiniger 2001). Within North America, the large number of vegetative incompatibility types within *C. parasitica* populations has prevented the natural spread of the hypovirus, and the application of the dsRNA hypovirus as a biocontrol agent has not been successful (Anagnostakis 1977; Anagnostakis 1981; Anagnostakis et al. 1986; Anagnostakis 2012).

Considerable effort has been directed towards lessening the effect of *C. parasitica* on *Castanea* species worldwide, including genetic engineering (of both host and pathogen), hybridization, and hypovirulence (Uchida 1977; Elkins and Griffin 1985; Anagnostakis 2001; Robin and Heiniger 2001; Diskin et al. 2006; Polin et al. 2006). However, despite the commercial and ecological importance of chestnut blight, surprisingly little is known concerning the mechanisms and metabolomics involved in the host-pathogen relationship between *C. parasitica* and *Castanea* species (Robin and Heiniger 2001). Metabolomics is a powerful tool that can allow researchers to identify and target key metabolites that either hamper the pathogen or increase natural host tolerance. This review describes known and suspected metabolites involved in host-pathogen interactions between species in the *Castanea* genus *and C. parasitica*, and where possible links those metabolites to known genes. This review also reports on abiotic and biotic factors which have been observed to affect the host-pathogen interaction between *C. parasitica* and *Castanea*. Anatomical evidence that distinguishes the tolerance mechanism in putatively tolerant compared with susceptible specimens is also described. By helping to fill knowledge gaps on chestnut blight infection, with a view to better understanding this devastating disease, it is hoped that more rapid

progress can be made towards the production of *Castanea* trees with greater levels of blight tolerance.

***Initiation and progression of chestnut blight in Castanea species***

The symptoms of chestnut blight vary in severity depending on the relative tolerance or susceptibility in the *Castanea* host species (Graves 1950; Mellano et al. 2012). In the most blight susceptible species, death of the above ground biomass by stem girdling is often rapid, while tolerant hosts can survive blight infection with only superficial symptoms. This section details the progression of *C. parasitica* disease in *Castanea* species. Where known, distinctions between blight susceptible and blight tolerant species are noted.

**External disease symptoms in *Castanea* hosts**

On the external periderm of the tree, blight presents as brown to black stem cankers, often covered with orange-yellow stromata approximately 1.0 – 2.0 mm in height and diameter (Uchida 1977; Hebard et al. 1984). Stromata breaking through undamaged periderm tissue are 1.0 - 1.5 mm in length, while stromata which emerge through cracks in the host periderm are larger, approximately 1.5 – 2.5 mm long and 1.5 – 2.0 mm in height. Pycnidia within the stromata emerge after a fresh rain, and produce spore horns with a stem-like appearance. Cankers are typically sunken, but may also appear swollen. Periderm directly on and around the canker may split or slough off (Griffin and Elkins 1986). If cankers are successfully sealed off by the host, the necrotic region of the canker generally falls off over time. Cankers are more visible on *C. dentata* and *C. sativa* than on Asian chestnut species, where cankers are often less severe and a more pronounced rhytidome (successive periderm layers, where the outer periderms may slough off) may obscure existing cankers. Hypovirulent strains of *C. parasitica* generally produce relatively smaller superficial cankers that do not reach the vascular cambium of the host (even on susceptible trees), and rarely produce stromata (Biraghi 1953; Grente 1965; Hebard et al. 1984; Griffin and Elkins 1986). The progression of canker growth has been best described in blight susceptible *C. dentata* and blight tolerant *C. mollissima* (Hebard et al. 1984). It is likely that chestnut blight progresses in a similar fashion in other *Castanea* hosts, albeit with differing levels of severity.

Initial infection proceeds with the appearance of a lesion on the periderm 8 – 11 days after inoculation. For the next approximately 15 days, no canker growth occurs. After this lag phase, the canker begins to expand at a steady rate until the tree is completely girdled, the host successfully sequesters the pathogen, or winter weather cuts short the growing season. Canker

growth can be rapid, particularly in susceptible trees. One study noted canker growth rates of approximately 0.85 mm/day in *C. dentata* (Hebard et al. 1984). The initial delay in canker growth has been associated with host defence responses, which are described in the following section. Both between and within species, the size and growth rate of the canker is taken to be a measure of disease severity; strains with greater virulence or hosts with greater susceptibility generally demonstrate faster canker growth (Powell et al. 2007).

Susceptible species employ various strategies to continue growth after blight infection. New epicormic shoots often form directly under the canker on the stem of an infected tree (Murrill 1906; Metcalf 1912; Anagnostakis 1987). If the total above ground biomass of the tree is killed by blight, the stump may continue to produce sprouts for several years (Mattoon 1909; Paillet 1984; Paillet 1988; Paillet 2002). These sprouts generally produce nuts for one or two seasons, before they too are girdled by blight. A stump will continue resprouting for many years, until the depletion of resources prevents further formation of sprouts. During the initial blight epidemic of *C. dentata*, it was observed that small trees (≤ 8 cm diameter at breast height; DBH) were usually killed within the initial year of infection, while mature trees took as many as 3 – 4 yr to die (Griffin and Elkins 1986). Blight is still considered nearly always fatal in *C. dentata*. There is evidence that relative tolerance may be developing in some local populations (particularly Canadian sub-populations), with a majority of infected individuals surviving blight infection for more than a decade (Van Drunen et al. 2017). However, mortality rates have been poorly documented over time, so it is difficult to determine if blight tolerance rates have improved, or are simply more closely monitored in recent years.

**Disease progression and host response**

External changes to the host have been relatively well studied compared with morphological and chemical changes within the host in response to disease progression. Although the following information is presented in great detail, it is important to note that many of these observations were made in only a few studies on a limited number of specimens. As such, the information presented here should be interpreted with caution.

*C. parasitica* enters the host by way of a wound on the epidermis or periderm (Uchida 1977; Hebard et al. 1984; Anagnostakis 1987). Insect damage is commonly believed to be the primary source of entry, although an insect vector is not obligate in *C. parasitica*. Damage by the chestnut gall wasp (*Dryocosmus kuriphilus*) has been identified as an entry point for chestnut

blight (Uchida 1977). Poorly healed graft unions in orchard trees of *C. crenata* have also been noted as an entry point. It is also known that infection can occur through lenticels; natural gaps in the periderm through which air exchange occurs. The rhytidome of large *Castanea* trees varies in thickness, even on a single tree, and parts of the outer periderm have been observed to slough off in some instances (Whitmore 1963). The quality (thickness and presence) of the periderm or the rhytidome has been suggested as playing a role in blight susceptibility (Hebard et al. 1984).

From the initial site of infection, the disease spreads outwardly within the host. In the early stages of infection, hyphae spread primarily intracellularly (Hebard et al. 1984) (Figure 5.1). Increased tannin concentration in the host can often be seen directly surrounding mycelial mats (Figure 5.2). The secondary phloem is generally infected before the cortex. The number of hyphae found in a lesion in the first few days of infection seems to differ depending on the natural blight tolerance of the host. Blight tolerant Chinese chestnut has been observed during infection to have half the number of hyphae seen in a susceptible American chestnut host. The host response to the pathogen typically becomes visible at the cellular level approximately 5 – 10 days after inoculation. As part of the initial host response, the host forms a zone of lignified cell walls around the lesion. This "lignified zone" is believed to consist of axial and ray phloem, and cortical parenchyma with lignified walls. Staining with phloroglucinol-HCl, safranin, and yellowish green fluorescence, has isolated the lignification to the middle lamellae, with deposition apparently initiating at the corners of the cell. Staining for peroxidase and β-glucosidase activity revealed that both of these enzymes were markedly more active in the lignified zone. The lignified zone ranges in width from 1 to 15 cells deep. After formation of the lignified zone, host cells trapped inside the lignified zone are killed by the infection while cells on the inner edge (towards the xylem) of the lignified zone remain metabolically active. It is believed that the delay in canker growth on the surface of the stem between initial lesion development and canker expansion occurs because of formation of the lignified zone within the host. The lignification of host cells initially delays the expansion of fungal mycelium. In blight tolerant trees, the pathogen is effectively sealed in and surrounded by this lignification. In susceptible trees, the mycelial fan is able to outgrow deposition of the lignified zone. When mycelial fans penetrate the lignified zone, host cells were sometimes observed to be neatly split by the hyphae. Hyphae also extended between the phloem fiber bundles in tangential layers, physically pushing aside and crushing young non-lignified interfascicular cells (Whitmore 1963; Hebard et al. 1984). Only the mycelial fan is capable of directly breeching the

lignified zone; individual hyphae or even aggregate groups of up to 8 hyphae could not penetrate. However, individual hyphae could still move beyond the lignified zone through gaps. If a fan is able to breech host defences in a tolerant host, generally only a single entry point is observed. In susceptible trees, multiple fans are often observed and are frequently distributed through multiple tissue layers of the stem, often reaching down to the vascular cambium (Hebard et al. 1984; Griffin and Elkins 1986). Where multiple fans occur in trees 6 yr or older, fans tend to be greater in size and closer to the outer edge of the periderm, giving the infection zone a "boat shape" appearance.

After the formation of the lignified zone, the host begins to form wound periderm to isolate the infection (Figure 5.2; Hebard et al. 1984). Tolerant trees (*C. mollissima*) begin wound periderm formation before susceptible trees (*C. dentata*); 10 – 14 days after initial infection. In contrast, susceptible trees take 14 – 48 days to initiate wound periderm. The orientation of the lignified zone has a direct effect on the initiation of wound periderm formation. Wound periderm formed next to periclinally-oriented cells usually begins to form twice as quickly as sites on anticlinally oriented cells. Susceptible trees were noted to have a higher frequency of anticlinally oriented infection zones. When wounding was made in periclinal portions of susceptible trees, wound periderm formation was apparently not delayed. Wound periderm typically begins in the secondary phloem closest to the initial inoculation site and develops towards the outer periderm. In blight tolerant trees, wound periderm completely envelops the lignification zone within 20 days. In healthy *C. dentata* trees (no blight inoculation), the formation of similar wound periderm in response to mechanical wounding required 30 - 40 days. The formation of wound periderm in blight infected *C. dentata* is not as well documented as in *C. mollissima*. In susceptible *C. dentata*, the expanding mycelial fan of *C. parasitica* interrupts wound periderm development. When fungal hyphae (in the form of mycelial fans) were able to breach the lignified zone and reach the wound periderm, the formation of wound periderm was interrupted by the death of the phellogen cells. Depth of periderm cells has been noted to be highly variable in *Castanea*, even within the same species (Whitmore 1963; Figure 5.3). The depth of the wound periderm varies depending on host susceptibility (Hebard et al. 1984). Wound periderm in tolerant hosts consists of up to nine layers of phellem cells and up to seven layers of phelloderm cells. In susceptible trees, these figures are seven and four respectively, approximately five cell layers less than in tolerant hosts. Susceptible trees also had a greater incidence of underdeveloped wound periderm (defined as periderm with gaps, or thin regions only two phellem cells thick).

Both xylem and phloem conductance is directly affected by blight infection, resulting in the death of tissues above the canker (McManus and Ewers 1990). The means by which *C. parasitica* affects xylem conductance is not conclusively known, although some mechanisms have been proposed. Many large tyloses and high concentrations of gums were observed in the outermost xylem tissues of infected *Castanea* (Bramble 1938; McManus and Ewers 1990). It was speculated that these structures and compounds were impeding water flow in *Castanea*, as occurs in *Prunus* spp. during *Cytospora* infection (Hampson and Sinclair 1973; Stanova 1985; McManus and Ewers 1990). Alternatively, fungal enzymes may be degrading vessel walls, resulting in vessel embolism and air-seeding (Newbanks et al. 1983; McManus and Ewers 1990).

***Hypovirulence and its role in determining virulence factors***

Hypovirulence in *C. parasitica* is caused by the infection of *C. parasitica* by several species of dsRNA hypovirus (Robin and Heiniger 2001). Several different strains of the hypovirus have been documented parasitizing *C. parasitica* across Europe and North America. When infected with a hypovirus, *C. parasitica* is still able to infect a *Castanea* host, but the resulting infection is much weaker and is often successfully contained by the wound periderm of the host. Hypovirulent strains are phenotypically very different from wild type strains and are simple to identify visually (Griffin and Elkins 1986; Kim et al. 2002). In vitro, hypovirulent strains generally have slower mycelial growth and a faded or white color compared to the relatively rapid mycelial growth and bright orange colour of the wild type strains (Figure 5.4). Strains infected with the hypovirus also generally display lower levels of conidia formation relative to the virulent strain. Infection with the hypovirus may also downregulate mating factor genes Mf2/1 and Mf2/2, which results in sterility in the female mating type (Kim et al. 2002).

Comparisons of DNA methylation profiles of virulent and hypovirulent strains during infection showed as many as 600 differently methylation regions (Li et al. 2018). Specifically, methylation in gene promotors in the hypovirus strain were negatively correlated with gene expression. As many as 144 functional genes are affected by the hypovirus, including genes involved in conidial formation, coloration, and virulence. However, many of these genes remain to be categorized, and it is unclear what role they may play during active blight infection.

The discovery of hypovirulence heralded an important turning point in the battle against chestnut blight. Not only did hypovirulence allow the European chestnut to remain commercially viable, it also provided the best method for identifying virulence factors in *C. parasitica*. Among

the following potential virulence factors described in this review, many were identified by comparing the metabolic profiles of both wild type and hypovirulent strains of *C. parasitica*, identifying and isolating metabolites lacking in the hypovirulent strain, and then testing these metabolites using in vitro or in situ assays on chestnut tissues. Much of the research into the metabolomics of *C. parasitica* would not have been possible without the discovery and application of the dsRNA hypovirus of *C. parasitica*.

**5.3. Overview of virulence factors**

In this review, mycotoxins from *C. parasitica* have been classified into one of four groups based on consensus in the literature supporting the pathogenic action of each compound within the *Castanea* genus (see Table 5.2). This grouping is based on the extant literature supporting the virulence of the compound alone, and should not be taken as an indication of the relative importance of each compound to the overall pathogenesis of *C. parasitica*. Compounds assigned to Group 1 have a pathogenic effect on *Castanea* tissues in vitro, and were identified during active blight infection in vivo. These compounds are very likely to be important, active virulence factors involved in the progression of chestnut blight. Group 2 compounds are mycotoxins with likely involvement in the pathogenesis of chestnut blight. They have proven toxicity to *Castanea* tissues in vitro and are well-documented virulence factors in other pathogenic fungi. However, Group 2 compounds have not yet been isolated during an active blight infection in vivo, and therefore have not been confirmed as being actively involved in blight infection. Group 3 compounds were lacking in hypovirulent strains relative to virulent strains of *C. parasitica*, and have plausible mechanisms through which they may affect virulence. Some Group 3 compounds are also known virulence factors in other host-pathogen interactions outside of *Castanea*. However, their effect on *Castanea* tissues has yet to be confirmed either in vivo or in situ. Group 4 compounds are suspected mycotoxins that demonstrate differing roles in virulence among other plant species, and have not yet been tested for virulence on *Castanea* tissues either in vitro or in situ. Group 4 compounds are the least likely to contribute to pathogenesis *in C. parasitica*, but may still play a role.

***Group 1: Confirmed virulence factors***

Group 1 contains the most important virulence factors known to be present during active blight infection. To date, only oxalate can qualify as a Group 1 virulence factor. Extensive research both in vitro and ex vitro has confirmed the specific toxicity of this compound to *Castanea* tissues.

Furthermore, it has been detected in the advancing edge of an active infection and its absence has been strongly linked to a loss of virulence in the pathogen.

**Oxalate**

Oxalate (oxalic acid), occurs as a free acid but is most often present as a calcium or potassium salt (Kirtzman et al. 1977). Calcium oxalate crystals take diverse forms in plants, and more than one crystal type may be found in a single organism (Franceschi and Nakata 2005). Oxalate was first identified in fungi by Hamlet and Plowright (1877), who found the compound in 27 fungal species. In pathogenic fungi, oxalic acid is a known key virulence factor (Dutton and Evans 1996). Virulence during host infection is either directly related to oxalic acid accumulation, or has a species-specific threshold relationship (Marciano et al. 1983; Punja et al. 1985). The role of oxalic acid in the pathogenesis of a fungal pathogen can manifest via different mechanisms, more than one of which may be operating in the host at the same time. Oxalic acid lowers the pH in host tissues, facilitating the operation of specific fungal enzymes with optimal function at a relatively acidic pH (Bateman and Beer 1965). In some species, oxalic acid works synergistically with polygalacturonase (PG). Oxalate chelates the calcium from the calcium pectate of the middle lamellae, facilitating the hydrolysis of pectates by PG. In several fungal pathogens, oxalic acid is produced before PG, supporting the primary role of oxalic acid in the infection process (Ikotun 1984; Punja et al. 1985). However, this is not the case in all species where oxalate and PG are believed to work synergistically. In vitro, oxalic acid production is affected by the concentrations of carbon, nitrogen, and the pH of the medium (Lapeyrie et al. 1987; Dutton and Evans 1996; Manteau et al. 2003; van Kan 2006). In some fungal species, oxalate production is stimulated in the absence of carbon or nitrogen sources, but supressed under high nutrient conditions (Dutton et al. 1993; Kuan and Tien 1993). It is unclear whether in vitro medium components may similarly affect growth during active infection in vivo, therefore, caution should be used when applying in vitro results to in vivo scenarios.

The mechanism through which oxalate affects successful *C. parasitica* infection has not been well studied. However, it is believed to be similar to most pathogenic fungi. As oxalate precipitates to form calcium oxalate crystals, two hydrogen atoms are released in the process, acidifying the surrounding intracellular space of the host (Griffin and Elkins 1986). With this mechanism, oxalate functions to reduce the intercellular pH of the host at the infection edge, promoting the action of fungal enzymes (McCarroll and Thor 1978a; Griffin and Elkins 1986).

Calcium and calcium oxalate are potentially involved in the regulation of both oxalate production and mycelial growth. In vitro, oxalate production in *C. parasitica* seems to be stimulated by calcium, while low concentrations of calcium actually suppress mycelial growth (Bennett and Hindal 1989). Mycelial growth also seems to be affected during the conversion process from free calcium to calcium oxalate. As oxalate is produced by *C. parasitica*, the free calcium is bound to calcium oxalate and mycelial growth increases (Englander and Corden 1971). However, as calcium oxalate accumulates, it seems to inhibit mycelial growth (Bennett and Hindal 1990). In vitro, the inhibition of mycelial growth by calcium oxalate can be overcome by the addition of $CuSO_4$, $FeCl_2$, or $FeCl_3$, which dissolve the calcium oxalate crystals. This relationship would further suggest that calcium oxalate has a direct effect on mycelial growth. However, other studies have shown that mycelial growth proceeds even when oxalate production has ceased (Bennett and Hindal 1989; Vannini et al. 1993). The relationship between free calcium, oxalate and mycelial growth needs to be investigated further.

Oxalate production has been sporadically studied within *C. parasitica*, and many unknowns remain. Oxalate is often found within *C. parasitica* as crystal calcium oxalate, although the exact crystal structure has yet to be described (Bennett and Hindal 1990; Figure 5.5). Oxalate production and mycelial growth do not correlate in *C. parasitica*. In vitro studies have documented calcium oxalate both in the medium directly surrounding mycelium, and in the hyphal walls of both aerial hyphae and hyphae in contact with the medium. Calcium oxalate in the in vitro medium is likely produced by oxalate exuding from the hyphae and complexing with free calcium ions in the medium to form crystals. In addition to free oxalate, oxalate crystals are found within aerial hyphae, suggesting that some oxalate crystals must form within the fungus. Within the hyphal wall, oxalate crystals can begin to form utilizing calcium from the fungus, and then emerge into the space around the fungal hyphae (Powell and Arnott 1985; Bennett and Hindal 1990). Several pathways for oxalate production are known to exist in fungi (Maxwell and Bateman 1968). However, it is not known which pathway is active in *C. parasitica*, or if there is any pathway redundancy (Bennett and Hindal 1990). Oxalate synthesis also seems to be pH-limited in *C. parasitica*, with in vitro studies noting greatest oxalate production in fungal hyphae when medium was adjusted to pH 5 (Bennett and Hindal 1990; Dutton and Evans 1996). However, in vivo studies have yet to confirm if this pH optimum holds during an active infection.

The importance of oxalate to the virulence of *C. parasitica* has been the best documented of all the potential virulence factors listed in this review. However, traditional in vitro studies and comparisons between hypovirulent and virulent strains showed conflicting results (Englander and Cordin 1971; Havir and Anagnostakis 1983; Chen et al. 2010; Zhang et al. 2013). Comparing oxalate accumulation between the hyphae of hypovirulent and virulent strains in vitro generally shows that oxalate accumulation is significantly reduced in the hypovirulent strain compared to the virulent control strain (Griffin and Elkins 1986; Bennett and Hindal 1989; Havir and Anagnostakis 1983; Havir and Anagnostakis 1985). Almost no detectable accumulation of oxalate occurred in hypovirulent strains in vitro (Havir and Anagnostakis 1983). At first, this suggested that oxalate was involved in the virulence of *C. parasitica*. However, in later studies it was noted that different levels of fungal oxalate accumulation occurred depending on both the infection by the dsRNA virus and culture environmental conditions (liquid or semi-solid media) (Bennett and Hindal 1989; Vannini et al. 1993). For instance, Vannini et al. (1993) noted that in liquid medium their hypovirulent strains of *C. parasitica* produced very low levels of oxalate compared to the virulent strains. However, when grown in non-host apple tissues, relative oxalate production rates were reversed and the hypovirulent strains produced much greater levels of oxalate relative to the virulent strains.

Although in vitro studies have given conflicting results, oxalic acid has provided researchers with some of the best in vivo evidence for the role of a virulence factor in the pathogenesis of *C. parasitica*. Null mutants of the oxaloacetate acetylhydrolase gene (which hydrolyses oxaloacetate to oxalate and acetate) in *C. parasitica* produced cankers that were only 10.0 % the size ($3.0 \pm 0.9$ cm$^2$) of those produced by wild type strains ($30 \pm 7.7$ cm$^2$) (Chen et al. 2010). Engineering blight susceptible *C. dentata* to more effectively break down oxalic acid has also produced results that hint at the importance of oxalic acid as a virulence factor in chestnut blight (Zhang et al. 1995; Zhang et al. 2013). The American Chestnut Research and Restoration Project has produced trees that are genetically engineered with an oxalate oxidase gene from wheat. Oxalate oxidase is a cell wall-embedded enzyme that converts oxalic acid into hydrogen peroxide and carbon dioxide, neither of which are harmful to *C. dentata*. Some of these genetically modified trees expressed as much as 200 times more oxalate oxidase than their wild type counterparts. Transgenic *C. dentata* lines producing the greatest levels of oxalate oxidase expression have been shown through stem inoculation assays to be highly tolerant to chestnut

blight compared with the wild type source trees. When inoculated with virulent fungus (strain EP155), canker growth was minimal and the disease never developed to a stage where it harmed the vasculature of the plant. To date, infected transgenic hosts have not been examined microscopically to determine the morphology of the host response relative to mycelial growth during infection. However, preliminary evidence with stem assays comparing relative tolerance in *C. mollissima*, to both wild type and transgenic *C. dentata* suggested a greater tolerance to chestnut blight in the transformed *C. dentata* than even the most blight tolerant Asian species of *Castanea* (Zhang et al. 1995; Zhang et al. 2013).

Despite conflicting studies in vitro, the in situ performance of genetically modified *C. parasitica* with impaired oxalic acid production suggests that oxalic acid has a very important role in the virulence of chestnut blight. These results also suggest a cautionary note when utilizing in vitro studies to interpret active blight infections in a living host. Much of the work investigating the metabolites involved in the host-pathogen relationship between *Castanea* and *C. parasitica* has been restricted to in vitro studies. These results must be confirmed in vivo before any final conclusions can be made from in vitro research.

***Group 2: Likely virulence factors***

Mycotoxins in Group 2 are implicated in the pathogenesis of chestnut blight. These are well-documented virulence factors in other pathogenic fungal species. In many cases, hypovirulent strains produce much lower concentrations of Group 2 compounds than do virulent strains. However, these compounds cannot be placed into Group 1 because they have not yet been documented in a *Castanea* host during an active blight infection.

**Tannase**

Tannase (tannin acyl hydrolase) is an enzyme common to several pathogenic fungal species (Iinuchi et al. 1972). It was first identified by Tieghem (1867) in *Penicillium glaucum* and *Aspergillus niger*. Tannase hydrolyzes the ester bonds of tannic acids, a common class of plant defence compounds (Farias et al. 1992). The greater the number of ester bonds, the greater is the tannase activity required to hydrolyze the substrate (Iinuchi et al. 1972; Farias et al. 1994). Tannase purified from *C. parasitica* functions optimally at a pH of 5.5 and a temperature of 30 °C (Farias et al. 1992). Enzyme activity can continue to a pH of 2.0 and at temperatures as low as 10 °C. The upper limit of enzyme functionality is at 55 °C, and can continue up to pH 8.5, where activity is minimal.

The role of tannase during blight infection, and its potential relationship to blight tolerance in *Castanea*, is complex and may involve end-product inhibition. Several studies have suggested that host tannins are used as a carbon source by the tannase of *C. parasitica* (Cook and Wilson 1915; Elkins et al. 1979). Tannase in *Castanea* hydrolyzes ester compounds of gallic acid, producing gallic acid and glucose. Intermediates identified in this reaction include 1,2,3,4,6-pentagalloyl glucose, 2,3,4,6-tetragalloyl glucose, and two unidentified compounds of monogalloyl glucose (Iinuchi et al. 1972). *C. parasitica* has been shown in vitro to utilize glucose as a carbon source (Bennett and Hindal 1989). However, gallic acid actually inhibits tannase activity (Haslam et al. 1961; Iinuchi et al. 1972). Farias et al. (1994) noted that in vitro, gallic acid as a substrate had the greatest inhibitory effect on purified *C. parasitica* tannase, suggesting feed-back inhibition. Gallic acid feed-back inhibition may be part of the natural pathogenic role of tannase. Tannase may have an important role in the early infection of *C. parasitica*. On culture medium containing tannins, levels of gallic acid peaked 3 days after *C. parasitica* inoculation and slowly decreased afterwards (Griffin and Elkins 1986). It could be that tannase is essential to initial cell degradation and is active on the mycelial edge of an active blight infection. *C. parasitica* may subsequently have a mechanism for supressing tannase production as other critical enzymes begin up-regulation. To date, developing cankers have not been examined for the presence of tannase. As such, the true role of tannase in blight pathology remains to be defined.

**Laccases**

Laccases belong to an exclusive group of enzymes characterized by four cupric ions, each associated with a single peptide chain (Choi et al. 1992; Chung et al. 2008). These are known as "blue copper oxidases" or "large blue copper proteins", and chiefly catalyze the oxidation of *p*-diphenols (Thurston 1994; Chung et al. 2008). To date over 100 distinct laccases have been purified and identified in fungi alone (Baldrian 2006). Unlike many fungal enzymes, there is significant structural and biochemical heterogeneity in fungal laccases across species. For example, laccase proteins can be found as both monomeric or homodimeric proteins, with pH optima ranging from 2 to 5. Some fungal species have multiple isoenzymes of laccase. There is also a group of enzymes with similar enzymatic activity as laccase, but which lack the cupric ions. Based on similar enzymatic activity, a minority of researchers consider these enzymes to be laccases, and term them "yellow" or "white" laccases to differentiate them from the blue colored

cupric laccases. However, most researchers consider only the cupric laccases to be true laccases. For this reason, only the cupric laccases are considered here (Baldrian 2006).

Laccases are found in select species of bacteria, insects, higher plants, and fungi (Baldrian 2006; Chung et al. 2008). The first fungal laccase was identified by Bertrand (1896). In pathogenic fungi, they are believed to be involved in a number of processes, many of which can be linked to virulence. These include: lignin degradation, hyphal growth, phytoalexin conversion, fruiting body development, spore pigmentation, and general fungal pathogenicity (Leonard 1971; Clutterbuck 1972; Ishihara 1980; Leatham and Stahmann 1981; Marbach et al. 1985; Geiger et al. 1986; Rigling et al. 1989; Lewis and Yamamoto 1990; Sengupta and Mukherjea 1997; Hataka 1998; Smith et al. 1998; Schouten et al. 2002; Chung et al. 2008). Direct evidence is lacking for many of these purported laccase activities (Rigling and van Alfen 1993). However, there is strong experimental evidence for conidial laccase of *Aspergillus nidulans* and secretory laccase of *Botrytis cinerea* (Clutterbuck 1972; Rigling and van Alfen 1993; Schouten et al. 2002). In *A. nidulans* for example, laccase is believed to be responsible for spore pigmentation, as laccase deficient mutants display yellow spores in comparison to the wild type green spores (Clutterbuck 1972).

Three laccases have been identified in *C. parasitica*, with indications that there may be a fourth (Kim et al. 1995). LAC 1 and LAC 3 are extracellular laccases (Rigling and van Alfen 1993; Kim et al. 1995). LAC 3 was discovered in null LAC 1 mutants by Kim et al. (1995), suggesting that LAC 3 may be a redundant laccase to LAC 1. Further studies showed that while LAC 1 is a constitutive laccase, LAC 3 transcription is only induced in the presence of tannic acid (Chung et al. 2008). In many fungi, laccases are encoded from gene families (Chung et al. 2008). With *C. parasitica*, LAC 1 and LAC 3 are coded from separate genes. LAC 2 is an intracellular laccase (Rigling and van Alfen 1993). LAC 1 and LAC 2 were found on separate genes, but it is unclear what the relationship is between LAC 2 and LAC 3 (Kim et al. 1995). In culture, laccase activity commences between 12 and 48 h after inoculation and peaks shortly afterwards. LAC 1 peaks at 72 h, while LAC 2 peaks at 96 h. LAC 3 activity generally peaks after 24 h, although one hypovirus strain tested had a delayed maximum which only occurred 3-4 days post inoculation (Rigling 1995; Chung et al. 2008). Laccase activity is also pH sensitive; the optimal pH of LAC 1 is 2.5, and activity declines as pH approaches 7 (Rigling and van Alfen 1993).

Strains of *C. parasitica* infected with the dsRNA hypovirus produced lower levels of laccase (Hillman et al. 1990; Rigling and van Alfen 1991). In particular, levels of LAC 1 and LAC 2 were 5 times less in hypovirulent strains compared to virulent strains (Rigling 1995). Hypovirulent strains are known to be deficient in specific characteristics which laccases are suspected of being involved in, such as sporulation (Rigling and van Alfen 1993). As well, LAC 3 was only transcribed in the presence of tannic acid, suggesting a role in pathogenicity in hosts containing tannic acid (such as *Castanea*) (Griffin 1986; Chung et al. 2008). Despite this, null mutants or mutants with laccase suppression have either shown no difference in virulence from wild types (LAC 1 and LAC 2 supressed mutant) or only a moderate reduction in virulence (LAC 3 null mutant) (Rigling and van Alfen 1993; Chung et al. 2008). This suggested the activity of an additional fourth laccase when LAC 1, LAC 2, and LAC 3 were supressed (Rigling and van Alfen 1993). Alternatively, it may be that very little laccase is actually required for virulent activities, so that laccase-supressed mutants maintain virulence on a level comparable to the wild type (Ringling 1995; Chung et al. 2008).

Laccase may also be working in conjunction with oxalate. Calcium oxalate is suspected of being an end-product inhibitor of oxalate synthesis (Vannini et al. 1993). Oxalate inhibition in the pathogen may be avoided when oxalate is diffused into the host tissues as far as possible from active mycelial growth. Even if calcium oxalate is not a true suppressor of oxalate production, its presence may facilitate the rapid spread of oxalate through host tissues to promote the activity or access to other fungal enzymes. For infection, oxalate must diffuse across the cell wall of the host. With virulent strains, laccase(s) degrade lignin, so oxalate diffuses away from the infection site. In hypovirulent strains, lesser laccase concentrations result in reduced lignin degradation and slower diffusion of oxalate occurs, resulting in suppression of pathogenesis. Laccase is very likely a significant factor in the virulence of *C. parasitica*. However, with multiple potential modes of action, more than one of which may be operating at any one time, more work needs to be done to fully elucidate the role of laccase in the pathogenesis of *C. parasitica* (Vannini et al. 1993).

**Polygalacturonases**

Polygalacturonases (PGs) are a group of cell wall degrading enzymes common to many fungal pathogens (Dutton and Evans 1996). Specifically, they depolymerise pectin from the middle lamella and cell walls of the plant host (Gao and Shain 1994). In plants, pectins are structurally diverse between species, tissues, and even within the same cell wall (Niture 2008). As a result,

PGs similarly vary widely in structure and biochemical composition between species. For example, although most PG have an acidic pH optimum which decreases sharply above pH 7, several bacterial species, including *Bacillus* and *Streptomyces* spp., can produce PGs that are stable to a pH of 9 (Kapoor and Kuhad 2002; Kuhad et al. 2004; Niture 2008). Similar to laccases, a single fungal species can produce several different forms of PG (Niture 2008). PG often acts in tandem with oxalic acid (Dutton and Evans 1996). Oxalic acid binds to the calcium of the calcium pectate in the middle lamella facilitating pectin hydrolysis by PG. In some fungal species such as *Sclerotium cepivorum*, PG is unable to hydrolyze pectate in the absence of oxalic acid (Stone and Armentrout 1985; Dutton and Evans 1996). In these cases, the virulence of the pathogen is often related to the concentration of oxalic acid produced in the host, and PG contributes very little to overall virulence. However, in other fungal pathogens, PG is more highly correlated with virulence than is oxalate (Punja et al. 1985).

In *C. parasitica*, the most well studied PG is an extracellular endopolygalacturonase produced by the gene *enpg-1* (Gao and Shain 1994; Gao et al. 1996). Given the likely importance of oxalic acid in the pathogenesis of *C. parasitica*, PG should presumably have an important role in pathogenesis as well. However, the relationship between oxalic acid, PG, and virulence in *C. parasitica* is complex. Hypovirulent strains of *C. parasitica* show reduced PG activity in vivo (Gao and Shain 1995; Gao et al. 1996). As well, lower PG activity occurred in the periderm of *C. mollissima* compared to *C. dentata* during infection. However, two *enpg-1* null mutants of *C. parasitica* failed to show any reduction in PG activity in vivo, despite absence of PG activity in vitro. As well, different substrate specificities and pH optima have been noted for the PG of *C. parasitica*. The PG of *C. parasitica* could not hydrolyze pectin in the absence of oxalate (Zhou et al. 1996). However, purified PG could hydrolyze isolated cell wall material from both *C. dentata* and *C. mollissima* in vitro (Gao and Shain 1995). The pH optimum for this activity was between 4.5 and 5.0 and activity decreased rapidly above pH 5.0, until inactivation at pH 6.0. However, others noted a slightly higher pH optimum for PG (5.0 to 5.5), and a similar pH optimum (5.3) for an unspecified pectinase (McCarroll and Thor 1985b). It was initially thought that *C. parasitica* had a single PG compound, with potential production of post-transcriptional isoforms (Gao and Shain 1994; Gao and Shain 1995). However, Gao et al. (1996) noted that their *enpg-1* null mutants synthesized two novel acidic PGs in vivo which were not observed in vitro. They further demonstrated that the hypovirus likely inhibited the activity in these two acidic PGs. This work,

in conjunction with other inconsistencies observed in pH and substrate specificity and the unspecified pectinase documented by McCarroll and Thor (1985b) suggest the likelihood of more than one form of PG involved in the pathogenesis of *Castanea* species. Unfortunately, previous studies have not differentiated between PG compounds and as a result, it is difficult to elucidate the true role of PG in virulence or precisely how its virulence relates to the presence of oxalic acid.

***Group 3 Possible or indirect virulence factors***

Group 3 compounds are found in lower concentrations in hypovirulent strains relative to virulent strains, and have plausible mechanisms that could explain their effect on the virulence of *C. parasitica*. In some cases, these compounds are known virulence factors in other host-pathogen interactions, although their role within *Castanea* is less certain. The role of Group 3 compounds in the host-pathogen interaction between *C. parasitica* and *Castanea* is unclear. However, there is a possibility that these Group 3 compounds may be involved in virulence, even if only indirectly.

**Cryparin**

Cryparin is a type II cell wall-associated hydrophobin (Zhang et al. 1994). Hydrophobins are small cell surface proteins with eight conserved cysteine molecules per compound (Kazmierczak et al. 2005). Hydrophobins are ubiquitous in filamentous fungi (McCabe and van Alfren 1999). The roles they perform vary widely between species. However, many are involved in modifying the hydrophobic properties of the cell surface (McCabe and van Alfren 1999; Kazmierczak et al. 2005; Kim et al. 2008). Monomer hydrophobins are secreted from cell walls through vesicles, and upon contact with a hydrophobic: hydrophilic interface undergo a structural change which enables an aggregation-prone region of the protein to make intermolecular connections with adjacent hydrophobin monomers (McCabe and van Alfren 1999; Macindoe et al. 2012). Together, monomers form an amyloid B-sheet over the cell surface and aggregate into insoluble rodlets that form an amphipathic monolayer on the cell surface. This layer provides additional desiccation protection for the surface of a fungal cell.

To date, the only fungal cryparin has been found in *C. parasitica*. During host infection, cryparin is exclusively found in the stromatal pustules of the fungus (Wessels 1997; Wosten 2001; Kazmierczak et al. 2005). However in vitro, cryparin was also found to be produced on vegetative aerial hyphae and to be secreted into the culture medium (Carpenter et al. 1992; McCabe and van Alfren 1999; Kazmierczak et al. 2005). Null mutants were used to demonstrate the necessity of this protein for the eruption of stromal pustules from under the host periderm (Kazmierczak et al.

2005). However, these null mutants showed no reduction in overall virulence within the host. Generally, hydrophobins require a hydrophobic:hydrophilic interface to assemble. However, McCabe and van Alfren (1999) observed that cryparin can bind to the cell walls of submerged hyphae in vitro, without a hydrophobic:hydrophilic interface. Cryparin also has many lectin-like properties which separate it from other hydrophobins, and these properties may allow it to bind to the fungal cell wall without these specific hydrophobic:hydrophilic interfaces. In vitro, cryparin is first secreted into the culture medium before associating with the hyphal cell wall. Secretion begins at the growing hyphal tip, and secreted cryparin is rapidly bound to the developing hyphal wall. It is also believed that cryparin secretion occurs in sections of the hyphal wall that are already saturated with bound cryparin. These cryparin-saturated wall sections are presumably older sections of hyphae that are most likely to further develop into stromata during active infection. However, these observations have only been made in vitro, and it remains to be seen if this same mode of action is present during an active infection in vivo. In *C. parasitica*, cryparin is transcribed from the *Crp* gene (Kim et al. 2008). It is the most abundantly transcribed protein in vitro, accounting for 22.0 % of total mRNA after 2 days in liquid culture (Zhang et al. 1994; Kim et al. 2008). In strains infected by the hypovirus, *Crp* transcription was drastically reduced. Comparisons between isogenic strains of virulent and hypovirus-infected *C. parasitica* showed *Crp* expression reduced by 50.0 to 70.0 % (Zhang et al. 1994; Kazmierczak et al. 1996).

Given the seemingly specialized role of cryparin in the reproductive cycle of *C. parasitica*, the compound is probably not directly involved in virulence. Null mutants of cryparin have reduced reproductive capacity, but no apparent reduction in virulence in host tissues.

**Cutinases**

Cutinases are a class of hydrolytic enzymes that specifically degrade plant cutins (Carvalho et al. 1999). Cutin is a polymer of hydroxyl and epoxy fatty acids that plays a key role in preventing the entry of pathogens into plants. Fungal cutinases are key to the enzymatic degradation of cutin, and are often the first and most crucial step in successful infection by a pathogen. However, some saprophytic fungi can also express cutinase(s), and some species can live indefinitely on cutin as a carbon source (Chen et al. 2013).

Cutinases are often a key virulence factor in fungal plant pathogens. However, only one study has examined *C. parasitica* for cutinases. An in vitro study comparing isogenic hypovirulent and virulent strains of *C. parasitica*, found suppressed levels of cutinase activity in the

hypovirulent strain (68.0 – 90.0 % less), and trace amounts of mRNA compared to the virulent strain (Varley et al. 1992). Unfortunately, this study did not examine cutinase expression during an active infection. It is widely believed that blight infection begins when *C. parasitica* enters the host through natural wounds (large or small) in the host periderm (Uchida 1977; Griffin and Elkins 1986). If this is the primary mode of infection, cutinase involvement in infection could be minimal as the pathogen would not need to degrade cutin to effect entry. However, cutinase presence in the virulent strain suggests this could be involved in hyphal entry into intact host tissues. Alternatively, *C. parasitica* can live as a saprophyte when there are no available hosts (Prospero et al. 2006). Perhaps the pathogen utilizes cutinase during these saprophytic growth phases in order to acquire carbon. Future studies should examine the role of cutinase in *C. parasitica* saprophytic and infective stages in more detail.

**Cellulases**

Cellulases are a group of extracellular fungal enzymes implicit in the pathogenesis of many fungal species. Along with polygalacturonases, cutinases, and proteases, cellulases are one of the cell wall degrading enzymes (Annis and Goodwin 1997). In particular, cellulase degrades the cellulose microfibrils in the primary cell wall by hydrolyzing the β-1,4 glucoside bonds in cellulose (Annis and Goodwin 1997; Bayer et al. 1998). As with the cutinases and PGs, cellulases come in a diverse range of structures and associated biochemical properties.

Very few studies have examined the role of cellulase in *C. parasitica*, and its involvement in pathogenesis remains to be determined. McCaroll and Thor (1985b) noted cellulytic activity in virulent strains of *C. parasitica* when grown in culture. In vitro, cellulase appears after 100 h in culture, and peaks between 250-300 h (depending on the strain), and has a broad pH optimum of 4.5 – 5.0. However to date, no cellulase activity has been found in active cankers of either *C. mollissima* or *C. dentata*, despite the presence of pectinase activity. Wang and Nuss (1995) identified and described a cellulase in *C. parasitica*. Encoded by the cellobiohydrolase gene *chb-1*, the cellulase is an unusual cellobiohydrolase I class enzyme that lacks a highly conserved cellulose-binding domain (Wang and Nuss 1995). Despite the lack of a cellulose-binding domain, in vitro studies have demonstrated that virulent strains of *C. parasitica* grown in media with cellulose as the sole carbon source had cellulase activity (Wang and Nuss 1995). Northern blot analysis showed the accumulation of *chb-1* transcript in these cultures, suggesting that cellulase activity is not merely the result of a second unidentified cellulase. In isogenic strains rendered

hypovirulent by dsRNA hypovirus, cultures did not secrete detectable levels of cellulase in vitro, and *chb-1* transcript accumulation was supressed (McCarroll and Thor 1985b; Wang and Nuss 1995). However, McCarroll and Thor did note cellulase activity in a hypovirulent strain in vitro (1985b). The *cbh-1* gene is likely only one of many as of yet unidentified cellulase genes present in *C. parasitica* that together could encode many cellulase enzymes with a range of substrate specificities (Wang and Nuss 1995). Unfortunately, it is unclear if they play any significant role in the pathogenesis of *C. parasitica*.

**Endothiapepsin**

Endothiapepsin is a rennet-like aspartic protease produced extracellularly by *C. parasitica* (Sardinas 1968; Choi et al. 1993). First identified in 1968, endothiapepsin was initially heavily investigated for clotting milk in a manner similar to rennet (Sardinas 1968). Endothiapepsin is maximally stable between pH 4 and 5, will denature at pH 7, and has a reported isoelectric point just below pH 4.5 or at pH 5.5 (Sardinas 1968; Hagemeyer et al. 1968). Further in vitro studies have shown a substrate specificity in endothiapepsin limited to peptide bonds involving amino acids with either neutral aliphatic or aromatic side chains (Williams et al. 1972).

In some pathogenic fungi, extracellular proteases have well documented and essential roles in pathogenesis (Ball et al. 1991; Mendgen et al. 1996). In *C. parasitica*, the role of endothiapepsin during blight infection has not been definitively determined. However, there have been some suggestive studies. The gene responsible for endothiapepsin synthesis was identified and its expression regulated to examine the potential role of endothiapepsin in virulence (Razanamparany et al. 1992; Choi et al. 1993; Allen et al. 2003). A mutant expressing a 7-fold increase in endothiapepsin compared to the wild type had a similar phenotype to the wild type in culture, but resulted in greater tissue necrosis in both chestnut phloem and xylem tissues in preliminary studies (Choi et al. 1993). As well, a transcriptional study comparing multiple hypovirulent and wild type strains of *C. parasitica* found infection with the dsRNA hypovirus resulted in downregulated transcription of both endothiapepsin and its precursor (Allen et al. 2003). Unfortunately, the role of endothiapepsin during infection of a virulent strain on chestnut tissues has not been studied. The current status of endothiapepsin as a virulence factor remains uncertain.

***Group 4: Tentative virulence factors***

In addition to more well documented mycotoxins of Group 3, some compounds have been identified in *C. parasitica* which are suspected mycotoxins (Bazzigher 1953; Boller et al. 1957;

Gaumann and Naef-Roth 1957; Gaumann and Obrist 1960; Gessler et al. 2013). These compounds have been purified from some pathogenic or otherwise saprophytic fungal species and shown to cause tissue browning or other deleterious effects in plant tissues. There is not definitive proof to claim that these compounds are virulence factors in any pathogenic fungal species. Nevertheless, these compounds have been identified in *C. parasitica* and have shown observed phytotoxic effects on plant tissues or differences in synthesis levels between virulent and hypovirulent strains of *C. parasitica*. These compounds include various polysaccharides, diaporthin, (+)-orthosporin, cryphonectric acid, and the anthraquinones chrysophanol, emodin, rugulosin, and skyrin (Bazzigher 1953; Boller et al. 1957; Gaumann and Naef-Roth 1957; Gaumann and Obrist 1960; McCarroll and Thor 1985a; Sparapano et al. 1989; Arnone et al. 2002).

**Polysaccharides**

Polysaccharides are generally considered to be a nutrient source for fungal species. However, with some fungal pathogens, specific fungal polysaccharides are phytotoxic and induce disease symptoms in both host and non-host species (Corasaro et al. 1998).

In *C. parasitica*, several extracellular polysaccharides were identified with phytotoxic activity (Corasaro et al. 1998). These include pullulan, and polysaccharide fractions including galactose, mannose, and possibly rhamnose (Corsaro et al. 1998). Pullulan is a polysaccharide consisting mainly of repeating units of a maltotriose trimer (Singh et al. 2008). Pullulan purified from *C. parasitica* caused yellowing and necrosis in stem and leaf tissues of *C. sativa* and in the non-host species tomato (*Solanum lycopersicum*) Corsaro et al. (1998). Although the particular polysaccharide compounds in the fraction were not definitively identified, yellowing and necrosis occurred in tissues of *C. sativa* and *S. lycopersicum*. Subsequent work identified individual compounds in the exopolysaccharide fraction first identified by Corsaro et al. (1998). These included a mannose nonasaccharide, a polymer consisting of rhamnose, galactofuranose and mannose, and a galactan consisting of galactofuranose residues (Corsaro et al. 1998; Molinaro et al. 2002; Zhang et al. 2003). However, it is unclear if these compounds also have phytotoxic activity. The mechanism for polysaccharide phytotoxiciy is not well understood. In some instances large polysaccharide compounds are not easily absorbed or distributed through the xylem of plant tissues, and may physically block or disrupt water transfer, leading to tissue death (Forabosco et al. 2006). Alternatively, exopolysaccharides may not be involved directly in tissue death. They are suspected to play a role in spore adhesion in some species, an important role in pathogenesis but

not one directly involved in host tissue death. No exopolysaccharides have yet been isolated from actively growing cankers, so the ultimate role of these compounds in blight infection is uncertain (Forabosco et al. 2006).

**Diaporthin**

Diaporthin was first isolated in *C. parasitica* by Bazzigher (1953), and its presence during infection was apparently associated with reduced canker growth. It has since been identified in a limited number of other fungal species, including the wood rotting fungus *Daldinia concertrica* (Lee et al. 2006).

The role of diaporthin in fungi on the whole has yet to be conclusively determined. Diaporthin inhibited the permeability of protoplasts in rhubarb (*Rhoeo discolor*), caused wilting in *S. lycopersicum* shoots, and possibly interfered with formation of wound periderm in *C. sativa* (Bazzigher 1953; Boller et al. 1957, Gauman and Naef-Roth 1957; Grentle and Berthelay-Sauret 1978, McCaroll and Thor 1985a). Diaporthin isolated from *C. parasitica* inhibited the growth of some species of bacteria and yeast (Boller et al. 1957). Sparapano et al. (1989) grew *C. parasitica* strains in vitro for 3 wk and noted that while a virulent strain produced diaporthin, a hypovirulent strain seemed not to. However, a later study in which *C. parasitica* was grown in vitro for only 14 days detected diaporthin, as well as many other potential mycotoxins, in a hypovirulent strain (Arnone et al. 2002). In active cankers, diaporthin has only been identified in completely necrotized tissues far from the advancing infection edge. Directly applying diaporthin to *C. sativa* cuttings was reported to induce severe symptoms in the leaf, but these symptoms were not characterized (Sparapano et al. 1989). Diaporthin may play a role in blight pathogenesis, but much more work needs to be done in order to elucidate this.

**(+)-Orthosporin**

(+)-Orthosporin is a potential fungal mycotoxin, characterized from the pathogenic fungal species *Drechslera siccans* (Hallock et al. 1988; Harris and Mantle 2001). Limited studies have shown that (+)-orthopsorin can cause foliar necrosis in amaranth (*Amaranthus spinosus*), *Digitaria* spp., *Echinochola crusgalli*, soybean (*Glycine max*), and maize (*Zea mays*) (Harris and Mantle 2001). Although tissue necrosis was documented, no studies to date have identified the mode of action or biochemical role of orthosporin in fungi. (+)-Orthosporin has been isolated from virulent strains of *C. parasitica* (Arnone et al. 2002). However, it is unclear what the role of orthosporin is in *C. parasitica*, or whether it contributes to pathogenesis in any way.

**Cryphonectric acid**

Cryphonectric acid was isolated and found to be produced in large quantities by in vitro cultures of *C. parasitica* (Arnone et al. 2002). At high concentrations (100 μM), cryphonectric acid inhibits growth in tomato seedlings. The mode of action for cryphonectric acid growth suppression has not been identified. However, one recent study examined the effects of a series of isobenzofuran-1 (3H)-ones; synthetic analogues of cryphonectric acid (Teixeira et al. 2013). The analogues caused interference with the photosynthetic apparatus of spinach (*Spinacia oleracea*). However, not all synthetic analogues performed similarly. For instance, some analogues inhibited ferricyanide reduction by decreasing the rate of electron transport by as much as 50.0 %. In the same study, particular analogues actually stimulated electron transport. Some analogues also depressed the growth of the cyanobacterium *Synechococcus elongatus* in culture. This study was conducted with synthetic analogues of cryphonectric acid, and therefore cannot be used to directly infer the mode of action cryphonectric acid may take in a *Castanea* host. To date, no study has examined the effect of this secondary metabolite on *Castanea* tissues, or its potential role during the infection process or disease cycle.

**Anthraquinones**

Anthraquinones are a group of quinoid compounds responsible for pigments in plants, fungi, and lichens (Gessler et al. 2013). In fungi, they often contribute to mycelial pigment in colors ranging from yellow, orange, to brown. Commercially, many anthraquinones are important as textile dyes, and surprisingly, some are effective semiconductors in microelectronics (Räisänen et al. 2001, Mamada et al. 2009; Gessler et al. 2013). In medicine, anthraquinones have shown both mutagenic and anticancer properties (Tikkanen et al. 1983; Huang et al. 2007; Gessler et al. 2013). Anthraquinones also exhibited a range of antimicrobial activities, including growth suppression in bacteria and fungi (Agarwal et al. 2000; Chukwujekwu et al. 2006; Gessler et al. 2013). Several important anthraquinones have been identified in *C. parasitica*. In other fungal species, these anthraquinones were potentially phytotoxic. However, it is not clear if they perform the same role in *C. parasitica*.

***Emodin***

Emodin is an anthraquinone monomer and the most common anthraquinone in fungi (Gessler et al. 2013). Many other anthraquinones are made up of one or more emodin monomers (Kawai et al. 1984; Anderson et al. 1988). In some plant species, emodin has documented

antibacterial activity (Chukwujekwu et al. 2005; Gessler et al. 2013). For example, emodin extracted from the roots of *Cassia occidentalis* inhibited *Bacillus subtilis* and *Staphylococcus aureus* (Chukwujekwu et al. 2005). Sparapano et al. (1989) noted that while a virulent strain was found to produce emodin as well as more complex polymers of emodin, a hypovirulent strain only produced emodin monomers. This is the only study to have examined emodin in *C. parasitica.*

***Skyrin***

Skyrin is a dimer of two emodin monomers (Kawai et al. 1984). Skyrin was first identified in 1954 as the pigment compound responsible for orange coloration in *Penicillium islandicum* (Howard and Raistrick 1954). Pathogenic activity in skyrin has been poorly investigated. However, skyrin has been found to interfere with protoplast permeability in *R. discolor*, and was toxic to *S. lycopersicum* (Grumann and Obrist 1960; McCaroll and Thor 1985a).

Skyrin was found in a virulent strain of *C. parasitica*, but not in a hypovirulent strain (Sparapano et al. 1989). Hypovirulent strains of *C. parasitica* typically look white or faded yellow color in vitro, very different from the vibrant orange color of virulent *C. parasitica* (Griffin and Elkins 1986). Given that skyrin has been documented as an orange pigment compound in other fungal species, hypovirulent strains in culture may lack skyrin. In vitro bark assays with purified skyrin have shown browning on the inner bark of both *C. dentata* and *C. mollissima* (McCaroll and Thor 1985a). Interestingly, *C. dentata* was noted to tolerate twice the concentration of skyrin compared to *C. mollissima*. In active cankers, skyrin has only been found far from the advancing mycelial front in completely necrotized tissues. Skyrin isolated from active cankers failed to cause browning in a bioassay with *Castanea* bark. In vitro, skyrin has only been found in sporulating mycelia. The lack of skyrin at the leading edge of the infection, as well as its presence only in the sporulating tissues of *C. parasitica*, suggest that skyrin does not play a primary role in the pathogenesis of *C. parasitica*. Nevertheless, it is a documented mycotoxin in other fungal species, and may still function during infection. More work needs to be done to investigate the specific action of skyrin (McCaroll and Thor 1985a).

***Rugulosin***

First isolated in 1939, rugulosin has a yellow coloration and along with skyrin, was considered to be a pigment compound of *P. rugulosum* (Breen et al. 1955). Strains appearing white in culture have little to no rugulosin, while strains with yellow to orange mycelium have rugulosin. Emodin is one of the breakdown products of rugulosin. Rugulosin may potentially be involved in

fungal defence against insect predation. Fungi producing rugulosin have been applied as a foliar spray for *Pinus* spp. to reduce the growth rate of the eastern spruce budworm (*Choristoneura fumiferana*; Miller et al. 2002; Miller et al. 2008).

Very limited work has been done with rugulosin in *C. parasitica*. A hypovirulent strain failed to produce rugulosin, while a virulent strain of the fungus did (Sparapano et al. 1989). It is very possible that the pale character of hypovirulent *C. parasitica* in culture is the result of the suppression of rugulosin as well as skyrin. In developing cankers, rugulosin has only been identified in completely necrotized tissues far from the advancing edge. As with skyrin, rugulosin was only found in sporulating mycelia but did cause browning on inner chestnut bark when tested in vitro (McCaroll and Thor 1985a). Both the blight susceptible *C. dentata* and the blight tolerant *C. mollissima* were equally susceptible to browning from rugulosin. As with the other anthraquinones listed here, more work needs to be done to determine if rugulosin performs a role in *C. parasitica* beyond pigmentation, or if pigmentation itself performs a role in virulence.

***Chrysophanol***

Chrysophanol is another compound which utilizes the monomer emodin as a building block (Anderson et al. 1988). Chrysophanol has been isolated in some fungal species, but is most well studied in plants (Qian et al. 2011). It has been isolated from some vegetables such as lettuce (*Lactuca sativa*) and bean (*Phaseolus vulgaris*) (Mueller et al. 1999). In plants, chrysophanol had antibacterial activity against *Bacillus stubtilis* and *Staphylococcus aureus* (García-Sosa et al. 2006). Very little research has been done on chrysophanol in fungi, and its role in fungi is currently not understood. Chrysophanol was identified in virulent, but not hypovirulent, strains of *C. parasitica* (Sparapano et al. 1989). Research is needed to examine the role of this compound in *C. parasitica* and other fungi as well as in plants.

### 5.4. Possible metabolites involved in host tolerance

Formation of the lignification barrier and wound periderm are well characterized host responses in *Castanea* species to *C. parasitica* infection. However, the metabolomics of the pathogenesis related proteins and other defensive compounds produced by *Castanea* during blight infection remains poorly understood. Explored below are the currently identified compounds implicated in blight tolerance in *Castanea* (Table 5.3). With the ongoing work of the Hardwood Genomics Project, this list will become more comprehensive in the years to come (Carlson et al. 2014).

***Tannins***

Tannin compounds in plants are divided into two groups delineated by structure and biochemical properties. These are hydrolysable tannins and proanthocyanidins. Hydrolysable tannins consist of a polyol carbohydrate center (often D-glucose) with an attached hydroxyl group esterified with either gallic acid or hexahydroxydiphenic acid (Haslam et al. 1961; Cooper and Rieske 2008). Hydrolysable tannins esterified with gallic acid are known as gallotannins and galloylglucoses, while those esterified with hexahydroxydiphenic acid are known as ellagitannins (Cooper and Rieske 2008). Proanthocyanidins, also known as condensed tannins, consist of flavonoid chains (Cooper and Rieske 2008).

Tannins are an important secondary metabolite in *Castanea*. Before tannins could be synthesized artificially, the *Castanea* genus was one of the most important commercial sources of tannins (Hergert 1983). Within *Castanea*, tannin profiles include a wide range of compounds and can vary considerably between species and even between individuals of the same species (Delen 1975). Early studies noted high levels of tannins in infected bark (Figure 5.2). Initially, it was believed that tannins constituted an induced defence in *Castanea*, as tannins are part of the antifungal and antimicrobial defence systems in many plant species (Uchida 1977; Griffin and Elkins 1986). However, Cook and Wilson (1915) suggested that tannins were consumed by *C. parasitica,* presumably as a source of nutrition. In vitro studies have observed *C. parasitica* to grow better when supplied with extracts of *Castanea* tannins in the growth medium (Anagnostakis 1992). Tannase is likely the fungal enzyme responsible for this improved activity (Elkins et al. 1979). Tannin profile differences between Asian *Castanea* species and *C. dentata* and *C. sativa* may explain some differences in blight tolerance between these two species groups (Elkins et al. 1979; Griffin and Elkins 1986; Cooper and Rieske 2008). Asian, European, and American species of chestnut have similar levels of the tannins vescalagin and castagalin. However, *C. dentata* and *C. sativa* have high levels of hamamelitannin, while Asian chestnut species have low or undetectable levels (Elkins et al. 1979). Farias et al. (1995) demonstrated in vitro that hamamelitannin is an excellent substrate for tannase, while vescalagin and castagalin are more resistant to tannase activity. It is possible that the relatively greater content of vescalagin and castagalin in relation to hamamelitannin in Asian chestnut species slows mycelial growth allowing the host to fight off the disease. In contrast, in European and American species the greater presence of hamamelitannin facilitates rapid mycelial growth. Other in vitro studies examining the effect of

*Castanea* tannins on mycelial growth have generally shown similar results (Uchida 1977; Nienstaedt 1953). An extract of unidentified tannic acid from *C. crenata* applied in vitro at a concentration of 0.1 % was shown to promote mycelial growth in *C. parasitica*, although greater concentrations suppressed mycelial growth (Uchida 1977). A separate study showed that *C. parasitica* growth was inhibited on PDA medium supplemented with 1.2 % purified tannin extract from either *C. dentata*, *C. crenata*, or *C. mollissima* (Nienstaedt 1953). This supports the work of Uchida (1977), who found that concentrations of tannic acid greater than 0.1 % inhibited mycelial growth. However, it is important to note that the majority of studies examining the effect of tannic acid on mycelial growth in *C. parasitica* do not identify the specific tannic acids they are testing (Anagnostakis 1992). Instead, they refer to them as generic tannic acid. Therefore, it is difficult to conclusively interpret their results to determine the role of particular tannic acids such as hamamelitannin during blight infection. Additional phenolic compounds, apart from tannins, may affect pathogenesis in *C. parasitica*. In their study of the histology of canker development in *C. parasitica*, Hebard et al. (1984) noted that in *C. mollissima*, esterase activity continued in host cells after infection but stopped completely in *C. dentata*. Esterases could be involved in reducing host tannin availability. For example, esterases could bind tannins to proteins or carbohydrates, reducing their availability for pathogen use.

As well as interacting directly with mycelial growth, tannins may be interacting with jasmonic acid (JA). Tannins in *C. dentata* and *C. mollissima* responded very differently to JA treatment (Cooper and Reiske 2008). JA is an important signalling compound in induced plant defence in many agricultural crops, although its role is poorly studied in woody plant species. When *C. mollissima* was treated with a 1 g/mL/leaf solution of JA, levels of both hydrolysable and condensed tannins did not change in either leaf or stem tissue. In contrast, when *C. dentata* was treated with the same concentration of JA, in most cases tannin compounds increased between 1.5 – 2.0 times background levels. Hydrolysable tannins nearly doubled in both stem and leaf tissues, while condensed tannins also doubled in stem tissues. If *C. parasitica* is able to utilize hydrolysable tannins as a nutrition source, a tannin spike induced by blight infection in *C. dentata* may actually facilitate infection by *C. parasitica*. Detailed metabolic analysis of *Castanea* species following inoculation with blight is necessary to confirm this theory. Tannins may also be interacting with pathogenicity factors other than tannase. Gao and Shain (1995) found that PG activity was inhibited in vitro by tannins extracted from *C. dentata* and *C. mollissima*. However, tannin extracts

from *C. dentata* showed greater inhibition of PG activity than extracts from the more blight tolerant *C. mollissima*. The actual contribution of PG to virulence remains unclear, so it is uncertain whether the relative differences in the suppression of PG activity between the two *Castanea* species is directly related to blight tolerance (Gao et al. 1996). Nevertheless, it is important to note that although host tannins may form a substrate for tannase, they may also be interacting with other fungal virulence factors in an inhibitory way. Tannins clearly play a large role in the pathogenesis of *C. parasitica*. However, that role has yet to be fully defined.

***Peroxidases***

Peroxidases are a large group of variable isoenzymes responsible for catalyzing the one-electron oxidation of several substrates, producing a peroxide (Passardi et al. 2007; Almagro et al. 2009). Peroxidases are widely distributed across plant species, and are also found in bacteria, fungi, and animal species. Plant, bacterial, and fungal peroxidases belong to the same superfamily, while animal peroxidases belong to a different superfamily (Welinder 1992). There are three classes of peroxidases within the superfamily of plant peroxidases (Almagro et al. 2009). Class I peroxidases consist of chloroplast and cytosol ascorbate peroxidases. Class II peroxidases are limited to fungi, and are resecretory manganese peroxidases. Class III peroxidases are secretory plant peroxidases, and the class most commonly linked to pathogen defence in plants. Class III peroxidases are also linked to many other housekeeping functions in plants, including auxin metabolism, phytoalexin synthesis, the cross-linking of cell wall components, lignin and suberin synthesis, and the metabolism of reactive oxygen species and reactive nitrogen species (Passardi et al. 2005). Peroxidases are well studied pathogenesis-related proteins (Almagro et al. 2009). They are most often expressed in direct response to the pathogen. They are most commonly employed to either physically limit the spread of the pathogen through peroxide-dependant cross-linking of cell wall components to form irreversible structural barriers, or to produce an oxidative burst through high levels of reactive oxygen and nitrogen species (Sasaki et al. 2004; Passardi et al. 2005).

Four distinct peroxidases have been identified in the stem and leaf tissues in *Castanea* (Havir and Anagnostakis 1998). These peroxidases have been identified through gel electrophoresis, but their structure and enzymatic activities have not been well characterized. Peroxidase activity is involved in nut browning in *C. mollissima*. However, it is unclear if this nut peroxidase is also expressed in the leaf or stem tissues (Gong et al. 2015). Stem and leaf

peroxidases were initially classified by their band location on the electrophoresis gel, and were referred to as peroxidase A, B, or C (Santamour et al. 1986). Later work partially re-classified these peroxidases according to the terms peroxidase 1, 2, 3, and 4 (Havir and Anagnostakis 1998). The presence of these peroxidases differs between species, within species, and across the growing season (Santamour et al. 1986; Anagnostakis 1991; Tongkun et al. 1993; Havir and Anagnostakis 1998). Studies examining peroxidase in *Castanea* species generally report very skewed sample sizes, with samples of American species consisting of dozens to a hundred specimens, while European species are generally represented by a dozen or fewer specimens, and Asian species represented by between a one and as many as 30 specimens (Santamour et al. 1986; Anagnostakis 1991; Havir and Anagnostakis 1998). Therefore the differences in peroxidase isozymes seen between species needs to take into account the relatively low sample size for non-American chestnut species. Across studies, *C. mollissima* generally had the greatest diversity of peroxidases, despite the lower sample size compared to American species. Peroxidases 1, 2, 3, 4 and the peroxidase identified as C by Santamour et al. (1986) were identified in *C. mollissima*, either singly or as mixtures consisting of two different peroxidase compounds. *C. dentata* profiles, as well as *C. pumila* and *C. ozarkensis*, contained mainly peroxidase 3. However, Havir and Anagnostakis (1998) noted that peroxidase 2 and 4 were present in all species tested, including the American species. European chestnut was found to have a high diversity of peroxidase isoenzymes as well, with specimens that had solely peroxidase 1, peroxidase 3, or both peroxidases 1 and 3 combined. Hybrids between Asian and American species had as high a diversity of peroxidase profiles as the Asian parents (Anagnostakis 1991).

In *Castanea*, the role of peroxidase has been poorly investigated. Santamour (1988) noted that differing peroxidase isoenzyme profiles between individuals of *C. mollissima* were responsible for graft incompatibility. In individuals with similar peroxidase banding patterns, the cambial bridge formed and vascular continuity occurred between rootstock and scion. Vascular continuity failed to develop when isoenzyme banding profiles were different between scion and rootstock. Given the importance of peroxidases in lignin formation and the formation of primary cell walls, a failure of these enzymes would result in a serious retardation in wound healing. Zhesen et al. (2000) noted that water stress elevated levels of peroxidase in *C. henryi* seedlings, as did external applications of BAP.

The pattern of peroxidase isozymes between blight susceptible and tolerant *Castanea* species, and the role of peroxidase as a host defense protein in many plant species, suggest that host peroxidase may be involved in the defence interaction between *C. parasitica* and *Castanea* (Havir and Anagnostakis 1998). Barakat et al. (2012) noted greater levels of peroxidase transcripts in diseased canker tissue compared to healthy tissues in *C. mollissima*. However, Dane et al. (2014) noted much greater number of transcript reads (26 compared to 1) of peroxidase in healthy blight susceptible *C. pumila* compared to blight tolerant *C. mollissima*. Havir and Anagnostakis (1998) noted similarly conflicting results between peroxidase activity and blight susceptibility. Infection with blight was found to reduce peroxidase activity in both *C. dentata* and *C. mollissima*, although Chinese trees demonstrated a smaller decrease. In *C. dentata*, physical wounding also decreased peroxidase levels. However, in *C. mollissima*, wounding resulted in little to no detectable decrease in peroxidase levels. It has been suggested that in American chestnut, *C. parasitica* may be able to inactivate peroxidases. Unfortunately, despite the potential role of host peroxidases in the host-pathogen interaction as suggested by this study, no subsequent studies have examined the role of peroxidase in host defense.

***Chitinases***

Chitinases are a group of enzymes that hydrolyse the glycosidic bonds of chitin (Collinge et al. 1993). In plants, chitinases are involved in pathogen/pest defence reactions; chitin is the main component of the cell walls of fungal species and the exoskeletons of insect pests. Chitinases may also play a role in plants during early embryo development, although this has not been well studied (Collada et al. 1992; de Jong et al. 1992; Kragh et al. 1996). In *Castanea*, numerous studies have shown abundant chitinases in nut tissues, particularly in seedling cotyledons (Collada et al. 1992; Collada et al. 1993; Allona et al. 1996; Gomez and Aragoncillo 2001). However, it is unclear if these play any role in blight resistance, as the blight is primarily a stem girdling disease.

In *Castanea*, most studies investigating the role of chitinase in blight infection have only involved *C. sativa* (Allona et al. 1996; Schafleitner and Wilhelm 1997; Schafleitner and Wilhelm 1998; Wilhelm et al. 1998; Schafleitner et al. 1999; Vannini et al. 1999). Vannini et al. (1999) isolated four proteins with chitin-binding regions from *C. sativa* plantlets, and demonstrated that three of the proteins inhibited the mycelial growth of *C. parasitica* in vitro. Interestingly, hypovirulent strains were shown to be more inhibited by these host chitinases than virulent strains. This may reflect differences in mycelial growth rates between hypovirulent and virulent strains,

and the nature of chitinase proteins (Vannini et al. 1999). The youngest developing fungal cells are believed to be the most vulnerable to enzymatic hydrolysis by chitinases, as mature hyphae develop thick walls covered with proteins and contain chitin cross-linked to other polysaccharides. The slow growth of hypovirulent strains suggests that developing hyphae are vulnerable to chitinases for a longer time than the more rapidly maturing hyphal walls of the virulent strain, and this may be reflected in the different inhibitory rates observed in the Vannini et al. (1999) study. Inoculation studies examining gene expression and protein accumulation in *C. sativa* plants in response to infection with either hypovirulent or virulent strains also suggested that gene expression and chitinase activity were both induced systemically by infection, and persisted through the 7 days of the study (Schafleitner and Wilhelm 1997; Schafleitner and Wilhelm 1998; Schafleitner et al. 1999). Chitinase activity was also found to be greater in trees inoculated with hypovirulent strains relative to virulent strains of *C. parasitica*. This suggests that the virus responsible for hypovirulence may be contributing to pathogen recognition or response in the host tree, not simply interacting with gene expression in the pathogen. In both *C. dentata* and *C. mollissima*, transcripts of several compounds expressing chitinase activity accumulated more in canker tissue than healthy stem tissue (Barakat et al. 2012). However, no further studies have investigated the role of chitinases in the pathogen defence of *C. dentata* or *C. mollissima*.

***Salicyclic acid and other phytohormone signals***

Salicyclic acid (SA) and its glucoside, β-O-D-glucoside, are associated with pathogen defence in numerous plant species (Schafleitner et al. 1999). SA in particular is believed to be an important activator of pathogen resistance genes (Schafleitner et al. 1999; Harfouche et al. 2008).

The possible role of SA in blight tolerance has been examined only to a limited extent, and mainly in *C. sativa*. European chestnut plantlets grown in vitro accumulated SA after inoculation with both virulent and hypovirulent strains of *C. parasitica* (Schafleitner et al. 1999). Relative to wounded control plants, inoculated plants accumulated significantly greater levels of both SA and its glucoside over a period of 7 days. Interestingly, trees inoculated with the hypovirulent strains accumulated significantly more SA than both control trees and trees inoculated with virulent strains. This study suggested that the hypovirulent strain of *C. parasitica* may enhance host response relative to the virulent strain, or that virulent strains may be supressing host response. Studies have shown a similar relationship between hypovirulence and host gene expression with chitinase genes (Schafleitner and Wilhelm 1998). Transcriptome analyses in C. *dentata* and *C.*

*mollissima* cankers also showed an activation of SA transcription in canker tissue (Barakat et al. 2012). More work needs to be done to more accurately determine the role of SA during blight infection, particularly in *C. dentata* and *C. mollissima*.

***Cystatins***

Cystatins (CsCs) are cysteine proteinase inhibitors involved primarily in seed germination and maturation (Abe et al. 1992). In addition to this primary function, CsCs have been linked to involvement in programmed cell death, insect pest defence and the inhibition of fungal pathogens (Pernas et al. 1998; Pernas et al. 1999; Solomon et al. 1999). However, most of these pathogen-defence activities involve embryo tissues, not mature trees.

In *Castanea*, CsCs have been identified in the nuts of *C. sativa* and *C. dentata,* and the vegetative tissues of *C. sativa*, *C. dentata*, and *C. mollissima* (Pernas et al. 1998; Pernas et al. 1999; Pernas et al. 2000; Connors et al. 2001; Connors et al. 2002; Barakat et al. 2012). A *C. sativa* nut CsCs showed activity against insects and specific pathogenic fungi including *Botrytis cinerea* and *Colletotrichum graminicola* (Pernas et al. 1998; Pernas et al. 1999). CsCs accumulated in the leaves of *C. sativa* in response to wounding, JA treatments, and fungal infections (*B. cinerea*), suggesting a role in pathogen response in adult trees (Pernas et al. 2000). A vegetative *C. dentata* CsC was expressed in both healthy and blight infected tissues (Connors et al. 2002). In both *C. dentata* and *C. mollissima*, the transcripts for multiple CsCs were expressed in canker tissue (Barakat et al. 2012). Unfortunately, none of the CsC compounds identified to date have been directly tested for potential antifungal properties. As well, none of these CsCs have been examined for their effectiveness in inhibiting *C. parasitica* in culture. It is unclear if CsCs play any significant role in blight pathogenesis.

***Other potential pathogenesis-related proteins***

Several additional proteins with unspecified activities are of potential importance in the defence response of *Castanea* species to *C. parasitica*, and may be pathogenesis-related proteins (PRPs). In Schafleitner and Wilhelm's (1997) inoculation study, both hypovirulent and virulent strains of *C. parasitica* induced $\beta$-1,3-glucanase production in infected *C. sativa* trees. Chu and Ng (2003) identified a thaumatin-like protein in the nuts of *C. mollissima* with apparent antifungal properties. This protein, termed mollisin, inhibited mycelial growth in *Gusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora piricola*. Another antifungal protein, castamollin, was identified from the nuts of *C. mollissima* which inhibited the same fungal pathogens as

mollisin (Wang and Ng 2003). A novel thaumin-like protein was identified in the cotyledons of *C. sativa* (Garcia-Casado et al. 2000). In vitro, this thaumin-like protein showed anti-fungal properties in response to *Trichoderma viride* and *Fusarium oxysporum*. However, these three novel proteins were not tested against *C. parasitica*, and it is unclear if these nut proteins are found in *Castanea* spp. plants at later growth stages.

Gao and Shain (1995) tested a proteinaceous extract from both *C. dentata* and *C. mollissima* which inhibited PG activity. The extract from *C. mollissima* was 15 times more effective in inhibiting PG activity than was the extract from *C. dentata*. Interestingly, this proteinaceous extract was also effective in inhibiting a PG from a *Cryphonectria* sp., but not a PG from a *Rhizopus* sp. (Gao and Shain 1995). Unfortunately, the specific compounds found in the proteinaceous extract were not reported. In a direct inoculation study of *C. sativa* trees with both hypovirulent and virulent strains of *C. parasitica*, trees infected with the hypovirulent strains produced greater overall protein activity and a greater number of individual compounds than trees infected with the virulent strain (Schafleitner and Wilhelm 1997). This suggests that the host trees are forming a greater pathogen response to the hypovirulent strain, and the hypovirulent strain may actually be facilitating the pathogen response of the host. Alternatively, the pathogen may be influencing transcript accumulation as opposed to inhibiting gene expression (Schafleitner and Wilhelm 2002). Proteomic work in blight infection is limited. However, current studies suggest that proteins not typically associated with pathogen defence may be playing a role in affecting the blight tolerance of *Castanea* species.

***Sugars and other carbohydrate sources***

Carbohydrates are largely the fuel behind all metabolic activities in plants (Kozlowski 1992). There are many different forms of carbohydrate which can be stored in a single plant, from simple sugars such as sucrose, to more complex carbohydrates such as starch. Often carbohydrate profiles vary widely between species and even between individuals of the same species. Carbohydrates are also utilized by the host plant in order to build a defence against pathogens. However, in some instances, host carbohydrates have been used by plant pathogens to facilitate infection (Killiny and Almeida 2009).

*C. parasitica* has been extensively cultured in vitro. This has led to a fairly broad understanding of the effects of different sugar and other carbon sources on *C. parasitica* growth in vitro. Different carbon sources have shown major effects on hypovirulence, mycelial growth,

and the production of important pathogenicity factors such as calcium oxalate (Bennett and Hindal 1989; Bennett and Hindal 1990; Dutton and Evans 1996). Preferred carbon sources have been observed to be strain-specific. Bennett and Hindal (1989) in their multi-strain in vitro study observed that both virulent and hypovirulent strains produced the most oxalate in medium supplemented with calcium polypectate. Oxalate production decreased when isolates were cultured on the reducing sugars glucose or fructose. Cheng et al. (2013) noted that of 6 carbohydrate compounds tested, *C. parasitica* used monosaccharides most efficiently, followed by disaccharides and finally amylose. In vitro, glucose promoted significantly greater growth in *C. parasitica* than other carbohydrate sources (Cheng et al. 2013). Glucose also directly stimulated PG activity. Gao and Shain (1994) noted that an endopolygalacturonase produced by *C. parasitica* was stimulated by a 1.0 % glucose solution. The inner periderm (phelloderm) of chestnut was shown to be naturally high in glucose, containing as much as 2.0 % glucose (Gao and Shain 1994). Glucose is also one of the end products when galloyl esters are hydrolyzed by the fungal enzyme tannase, suggesting that tannase and PG activity may work synergistically (Iibuchi et al. 1972). It is unclear to what extent in vitro studies can be used as an indicator of virulence in vivo. No studies have yet investigated carbohydrate composition between *Castanea* species. However, it is possible that differences in carbohydrate profiles between *Castanea* species may be contributing to relative host tolerance or susceptibility.

***Possible defensive metabolites identified as part of the Hardwood Genome Project***

As part of the ongoing Hardwood Genome Project, a large scale transcriptome analysis was conducted on healthy and cankerous tissues from both *C. dentata* and *C. mollissima* (Barakat et al. 2009; Barakat et al. 2012; Carlson et al. 2014). While this analysis provided some insight into the defence mechanisms of *Castanea*, no obvious gene candidates responsible for the differences in blight tolerance between species were reported. It is also important to note that samples taken from healthy and infected canker tissues came from separate populations in Vermont and Connecticut, respectively (Barakat et al. 2009). It is possible that some differences in transcriptomes seen in healthy and diseased tissues are attributable to different genotypes and growing conditions. In canker tissue, the most frequent transcripts were hydrolyses, transferases, protein binders, and transporters (Barakat et al. 2012). As well, many genes involved in both local and systemic responses were identified (Barakat et al. 2009). These included genes involved in hypersensitivity, ATPase, the JA pathway response, lignin biosynthesis, laccase, polyphenol

oxides, and metabolic flux. Genes and transcripts with hydrolyse activity were the most represented of all protein groups. It was suggested that these hydrolyses may be involved in either strengthening cell walls as a defensive mechanism or in programmed cell death in the host. Kinase activity was also well represented in the transcriptome of both species. Kinase genes are involved in signalling during pathogen infection and often play a central role in the host response to the pathogen. Genes for RNA or protein binding were also well represented, and may be involved in the modulation of resistance genes in response to pathogen infection. Overall, the gene profile activated in canker tissues by *C. parasitica* was very similar between species (Barakat et al. 2009; Barakat et al. 2012). However, there were some measurable differences. In *C. dentata* more housekeeping genes were activated by *C. parasitica* and in *C. mollissima* more specific pathogenesis-related transcripts were observed, such as oxidoreductase (Barakat et al. 2012). For a comprehensive list of the transcripts identified, the reader is invited to read the texts of Barakat et al. (2009, 2012), which are too extensive to summarize completely in this review.

This transcriptome study highlighted how complex the host defence response is to *C. parasitica*. It is possible that the difference in host tolerance observed between *C. dentata* and *C. mollissima* will depend more on the speed of the host's response to the pathogen, as opposed to specific defence genes (Barakat et al. 2012). As well, resistance may be the result of the interaction between hundreds of genes, which is very difficult to elucidate through a transcriptome analysis. The most obvious physical symptom of the blight disease has always been a visible stem canker. In blight tolerant individuals, the pathogen is effectively sealed-off or isolated within a lignified zone by the host. In susceptible individuals, the mycelial fan of the pathogen is able to effectively spread beyond the lignified zone formed by the host. The transcripts found in the cankers of *C. dentata* and *C. mollissima* seem to confirm the importance of this process in host defence (Barakat et al. 2009; Barakat et al. 2012). Canker tissues in this analysis had high concentrations of hydrolases, transcripts involved in lignin synthesis, and transcripts involved in cell death from a hypersensitivity response. All of these processes would be necessary for the sequestration of a pathogen behind specific non-living host tissues. However, it remains to be seen which of the genes (or combinations thereof) identified in these transcriptome studies are crucial to blight tolerance. These large scale transcriptome studies are revealing, but in vitro and in vivo work examining transcript expression during active canker growth needs to be done to determine the relative importance of these canker-specific transcripts to the blight tolerance of each species.

## 5.5. Biotic and abiotic environmental factors potentially impacting host-pathogen interactions

***The immediate environment***

Many abiotic factors can work to either strengthen or weaken a host's defense during pathogenic attack (Anagnostakis 1982; Gomez and Aragoncillo 2001). With chestnut blight, various abiotic factors affect the severity of the disease in *Castanea* species, even in the more blight tolerant Asian species (Turchetti and Maresi 2008). Inter- and intra-specific competition has an effect on mortality from blight in *C. sativa*, *C. dentata*, *and C. crenata*; increased competition results in greater mortality (Uchida 1977; Griffin 2000; Turchetti et al. 2008). Inclement weather conditions can also increase blight incidence. For example with *C. crenata*, stressors including drought and cold stress increased blight incidence in orchard trees (Uchida 1977; Griffin and Elkins 1986). In *C. dentata*, canker growth rates were affected by season, with growth rates greatest in the warmer mo of July and August, and slowest in the cooler mo of June, September, and October (Anderson and Babcock 1913; Griffin and Elkins 1986). With *C. dentata,* severe frosts and drought were linked to increased blight occurrence, while with *C. sativa* hailstorms, wildfires, and drought were linked to increased blight incidence (Leonardi et al. 1995; Barthold et al. 2004; Turchetti and Maresi 2008). In *C. sativa* populations, these increases in disease occurrence were only temporary (Turchetti and Maresi 2008). Even in the very blight tolerant *C. mollissima*, cold stress was tentatively linked to increased blight incidence or canker growth (Berry 1954; Jones et al. 1980; Griffin and Elkins 1986). Shading has also been linked to increased blight susceptibility in *C. dentata*. Individuals of *C. dentata* grown in shade had a rhytidome that was absent in similarly sized trees grown in full sun.

Nutrient stresses have also been found to contribute to blight incidence. In *C. crenata* orchard trees, nitrogen deficiency was the most important factor contributing to blight incidence, having a greater effect on blight incidence than adverse climate conditions or intraspecific competition (Uchida 1977; Griffin and Elkins 1986). Alternatively, abiotic stress may activate compounds with inherent antifungal activities, making it more difficult for pathogenic fungi to begin infection (Gomez and Aragoncillo 2001). For example, in ungerminated *C. sativa* seeds, cotyledons exposed to thermal stress or microbial infection produced high levels of an endochitinase (CsCh3), a suspected defence protein in *Castanea*. Similarly, high levels of cystatin were produced in leaves of *C. sativa* in response to cold and saline stress (Pernas et al. 2000).

Although there is likely a connection between environmental factors and blight tolerance in *Castanea*, it is unclear which host mechanisms are affected by the environment. As well, it is unlikely that these factors could be harnessed to increase blight tolerance in the host.

***Endophytes and epiphytes***

Microbial ecology is complex, and often a parasite-host interaction exists simultaneously with competing microbial interactions within the host. In some cases, unrelated microbial species may either help or hinder an ongoing pathogenic infection. It was noted in several studies that some endophytic and epiphytic bacteria associated with *Castanea* and other plant species can affect the pathogenesis of *C. parasitica* in *Castanea* species (Wilhelm et al. 1998; Double et al. 2014). Some saprophytic fungal species (*Trichoderma* spp.) which have been isolated from existing blight cankers are known biocontrol agents, and may inhibit *C. parasitica* (Double et al. 2014). The presence of these species increased with the age of the canker, as did the presence of hypovirulent strains of *C. parasitica.* Eighty-two different bacterial isolates associated with *C. sativa* were examined for inhibition of *C. parasitica* and blight infection (Wilhelm et al. 1998). When co-cultured with *C. parasitica*, 40 different bacterial isolates were found to supress mycelial growth, of which 12 of these were isolated from the xylem of *C. sativa*, where they could theoretically come into direct contact with *C. parasitica* during infection. The strain most effective at inhibiting *C. parasitica* growth, both in vitro and on dormant stems of *C. sativa,* was identified as *Bacillus subtilis*. Interestingly, *B. subtilis* seems to be effective as a systemic treatment. *C. sativa* trees inoculated with this bacteria 1 wk before infection by *C. parasitica* showed significantly smaller lesion growth compared to control trees. Inoculation with *B. subtilis* at the same time as *C. parasitica* and at 2 wk before *C. parasitica* demonstrated low levels of inhibition or did not inhibit *C. parasitica* at all. In vitro micropropagated shoots of *C. sativa* and a *C. sativa x C. crenata* hybrid were also inoculated with *B. subtilis* and exposed to *C. parasitica*. Inoculated shoots showed similar beneficial results compared to in vivo trials, with shoots exposed to *B. subtilis* at 1 wk showing the greatest protective effect. Shoots treated with *B. subtilis* at 2 wk showed weak protective effects in culture. Most interestingly, the protective effects of *B. subtilis* survived through a round of subculture, although the resulting shoots had a significantly reduced micropropagation rate compared to un-inoculated shoots. Mechanistically, it was found that inoculation with *B. subtilis* promoted the transcription of an acidic extracellular chitinase and an acidic β-1,3 glucanase isoenzyme in the *C. sativa* shoots. The promotion of these pathogenesis-

related proteins, as well as the potential production of antifungal compounds by *B. subtilis* itself, likely contributed to suppression of *C. parasitica*. Unfortunately, the degree of promotion of these pathogenesis-related proteins and level of resulting fungal suppression was not described (Wilhelm et al. 1998).

Several unusual studies involving the direct application of soil to active cankers suggested that some soil microorganisms may also inhibit *C. parasitica* (Griffin and Elkins 1986). Mixtures of peat, muddy soil, or a mix of sphagnum and peat were placed over active cankers as a compress and held in place by either polyethylene or paper sleeves (Weidlich 1978; Griffin and Elkins 1986). Blight control and healing were observed on cankers exposed to muddy soil but not autoclaved soil over the 3-6 mo period of the study (Weidlich 1978). This suggests that some member(s) of the microbial community found in healthy soil may contribute to the suppression of *C. parasitica* at the canker site. Numerous species of bacteria, fungi, and actinomycetes were documented in the muddy soil used in this trial, although none were conclusively identified. More recent studies have identified several strains of the soil bacteria *Hypocrea rufa*, *Trichoderma viridie*, and an unidentified *Trichoderma* spp. which antagonized the growth of *C. parasitica* both in dual-culture and in vivo systems using cut logs of *C. sativa* (Arisan-Atac et al. 1995). However, all species investigated are native soil borne bacterial species, and it is uncertain if they could provide *Castanea* any protection against *C. parasitica* under natural conditions.

**5.6. Conclusions**

Chestnut blight has been referred to as one of the worst forest pathogens in recorded history (Hepting 1974). Its effect on North American chestnut species has been devastating. Only the natural emergence of hypovirulence in Europe and the widespread application of the hypovirus has saved the European chestnut from sharing the same fate (Anagnostakis 2001; Anagnostakis 2012). Even Asian species, which have presumably coevolved with *C. parasitica*, can be negatively affected by blight infection (Yan et al. 2007). Through this review, it is clear that although much work has been done, there are still considerable gaps in knowledge concerning the host-pathogen interaction of this disease within the *Castanea* genus. Oxalate is directly implicated in the successful infection of a host with *C. parasitica*. However, it is also the most well studied, and it may be that other virulence factors play an important role. The role of tannase, and particularly the end-product inhibition which may be regulating it during active infection, bears investigation. Both laccase and polygalacturonase need to be investigated for the possibility of

multiple isozymes within *C. parasitica*, and for their potential synergistic role with oxalate during blight infection. Cryparin may play a role beyond the emergence of stomatal pustules in *C. parasitica*. Both cutinase and cellulase are well documented virulence factors in other fungal pathogens, but have only been studied a limited number of times in *C. parasitica* (1 and 2 studies, respectively). The role of endothiapepsin needs to be determined in its effects on virulence in vivo. Although several fungal carbohydrates cause tissue browning in vitro, it needs to be determined if this indicates virulence in vivo. Diaporthin, (+)-orthosporin, and cryphonectric acid all demonstrate virulence in non-*Castanea* species, and need to be investigated more closely for their role in virulence with *Castanea*. Emodin and chryphanol have only been determined to vary between hypovirulent and virulent strains. They need to be tested in vivo with *Castanea* tissues. Skyrin and rugulosin are likely only involved in pigment in *C. parasitica*. However, their role in the virulence of other plant species may indicate a role in blight infection, even if only a minor one.

Decades of breeding work has been undertaken in order to increase the natural blight tolerance of *Castanea* species. However, these programs have never actively characterized possible host traits associated with increased blight tolerance. This review has identified several constitutive factors which may distinguish blight tolerant from blight susceptible individuals, including the natural thickness of the periderm along with the number of outer layers of suberized cells, the rate of growth of successive periderms within the rhytidome in response to infection, and the tannin profile in cells isolating the fungus. In addition, it has been noted that the dsRNA hypovirus may be increasing the speed of pathogen recognition in the host, particularly through salicyclic acid, resulting in greater expression of host chitinases. However, many of these studies examined a limited number of tree specimens, and a much larger sample size should be examined. As well, local abiotic and biotic factors have been shown to affect the host-pathogen relationship between *C. parasitica* and *Castanea* spp. As expected, any factors which stress trees (including inclement weather, inter- or intraspecies competition, and nutrient deficiencies) increase blight occurrence and progression. Inter-species competition on the microbial level has also been noted to increase host survival rate when infected with blight.

We are approaching a time of understanding and ultimately controlling the effect of *C. parasitica* on the *Castanea* genus (Griffin 2000). Modern genomics and genetic engineering have opened a path to saving *C. dentata* and other North American *Castanea* species. With the

Hardwood Genomics Project, it may finally be possible to take a close look at the genetics involved in blight tolerance and susceptibility in all species of the *Castanea* genus (Carlson et al. 2014). As well, the backcross breeding program conducted by the TACF is just now testing some of their most blight tolerant *C. dentata* x *C. mollissima* hybrids (Steiner et al. 2017). The American Research and Restoration Project of SUNY-ESF, also associated with the TACF, have engineered some remarkably blight tolerant lines of *C. dentata*, a trait that has been proven to be heritable (Newhouse et al. 2014). In Canada, the government of Ontario and the CCC are currently involved in a reforestation project designed to clonally propagate the remaining Canadian germplasm and produce seed orchards which would allow *C. dentata* to form self-sustaining populations throughout its native Canadian range (Boland et al. 2012). Other projects are focusing on reducing the virulence or transmission of *C. parasitica*, including engineering strains to facilitate the transfer of the dsRNA hypovirus, creating "super donors" which can reproduce with every mating type and potentially spread the hypovirus without regard for mating type (Zhang and Nuss 2016). It has taken more than a century, but it's possible that a disease which devastated species in the 20$^{th}$ century, may finally be controlled in the 21$^{st}$ century.

**5.7. Acknowledgements**

The authors are grateful for the Fonds de recherche du Québec - Nature et technologies (FQRNT) scholarship to C-AL and NSERC Discovery Grant support to DJD. We would like to thank Dr. Greg J. Boland, Professor Guelph University, for providing the stained and mounted slides used to create the figures in this review and the image of *C. parasitica* in culture. We are particularly grateful for valuable editorial input of Dr. Sandra L. Anagnostakis, Emeritus Scientist of the Connecticut Agricultural Experiment Station and Mr. Erik Carlson, Research Assistant (for Dr. Powell) at the American Chestnut Research and Restoration Project of SUNY-ESF and the TACF.

**Table 5.1**. *Castanea* species.

| Species | Section[1] | Common name(s) | Geographic Location | Nut(s) per bur | Commercial Use | Class of blight tolerance[2] | Reference |
|---|---|---|---|---|---|---|---|
| *C. crenata* | Eucastanon | Japanese chestnut | Japan, Korea | 3 | Nut | II | Mellano et al. 2012 |
| *C. mollissima* | | Chinese chestnut | China | 3 | Nut | I | |
| *C. seguinii* | | Dode's chestnut | China | 3 | Firewood | II, III, IV | |
| *C. sativa* | | European chestnut<br>Sweet chestnut | Europe, Asia Minor, North Africa | 3 | Nut, timber | IV | |
| *C. dentata* | | American chestnut | Canada, USA | 3 | Nut, timber | V | Paillet 1993<br>Mellano et al. 2012 |
| *C. henryi* | Hypocastanon | Willow leaf chestnut<br>Pearl chestnut | China | 1 | Timber | IV | Mellano et al. 2012 |
| *C. pumila* | Balanocastanon | Allegheny chinquapin | Southeastern USA | 1 | Timber, rootstock | III | Paillet 1993<br>Johnson 1988<br>Fu and Dane 2003<br>Mellano et al. 2012 |
| *C. ozarkensis* | | Ozark chinquapin | Arkansas, USA | 1 | Timber, rootstock | III, IV | Paillet 1993<br>Dane and Hawkins 1999<br>Johnson 1988<br>Fu and Dane 2003<br>Dane et al. 2003 |

1. *Castanea* species were divided into three sections by Louis-Albert Dode in 1908, based on nut shape and the number of nuts per burr (Johnson 1988; Lang et al. 2006). Eucastanon has 3 nuts per burr. Balanocastanon and Hypocastanon both have 1 nut per burr; with longer nuts in the Balanocastanon than Hypocastanon group.
2. As defined by Graves (1950), Class I presents with no fungal growth after inoculation, Class II is defined by slight fungal growth following complete healing of the canker, Class III is defined by a slow growth of the pathogen with callus formation (although the callus may be broken through by the pathogen), Class IV demonstrates rapid fungal growth with no callus formation, and Class V presents the most rapid fungal growth and no callus formation. Note that these classes are relative and were conducted on small sample sizes. Blight resistance in the overall meta-population will likely vary considerably. Nevertheless, Asian species of *Castanea* which have coevolved with the pathogen are relatively more tolerant to the pathogen than naïve European and American *Castanea* spp.

**Table 5.2.** Grouping of potential *C. parasitica* mycotoxins supported by existing literature.

| Grouping | Compound | Known Action | Hypovirulence | References |
|---|---|---|---|---|
| Group 1 | Oxalate | Lowers pH in host tissues<br>Chelates calcium from the calcium pectate of the middle lamella | Decreased to absent | Englander and Corden 1971<br>Havir and Anagnostakis 1983<br>Marciano et al. 1983<br>Havir and Anagnostakis 1985<br>Powell and Arnott 1985<br>Griffin and Elkins 1986<br>Bennett and Hindal 1989<br>Bennett and Hindal 1990<br>Vannini et al. 1993<br>Zhang et al. 1995<br>Dutton and Evans 1996<br>Chen et al. 2010<br>Zhang et al. 2013 |
| Group 2 | Tannase | Hydrolyzes ester bonds of tannins<br>Produces glucose as an end product | Not documented | Cook and Wilson 1915<br>Haslam et al. 1961<br>Iinuchi et al. 1972<br>Elkins et al. 1979<br>Griffin and Elkins 1986<br>Bennett and Hindal 1989<br>Farias et al. 1992 |
| | Laccase | Lignin degradation<br>Hyphal growth<br>Phytoalexin conversion<br>Fruiting body development<br>Spore pigmentation | Decreased | Griffin 1986<br>Hillman et al. 1990<br>Rigling and Van Alfen 1991<br>Rigling and van Alfen 1993<br>Kim et al. 1995<br>Rigling 1995<br>Chung et al. 2008 |
| | Polygalacturonase | Depolymerizes pectin from middle lamella and cell walls | Decreased | McCarroll and Thor 1985b<br>Gao and Shain 1994<br>Gao et al. 1996<br>Zhou et al. 1996 |
| Group 3 | Cryparin | Eruption of stromatal pustules | Decreased | Carpenter et al. 1992<br>Zhang et al. 1994<br>Wessels 1997<br>McCabe and van Alfren 1999<br>Wosten 2001<br>Kazmierczak et al. 2005 |

| | | | | |
|---|---|---|---|---|
| | | | | Kim et al. 2008 |
| | Cutinase | Degrades cutin | Decreased | Varley et al. 1992 |
| | Cellulase | Degrades cellulose microfibrils in the primary cell wall | Unchanged or decreased | McCaroll and Thor 1985a<br>McCaroll and Thor 1985b<br>Wang and Nuss 1995 |
| | Endothiapepsin | Protease limited to peptide bonds involving amino acids with either neutral or aliphatic aromatic side chains | Decreased | Razanamparany et al. 1992<br>Choi et al. 1993<br>Allen et al. 2003 |
| Group 4 | Pullulan | Results in tissue necrosis through unknown mechanisms | Not documented | Corsaro et al. 1998 |
| | Unnamed mannose nonasaccharide | Results in tissue necrosis through unknown mechanisms | Not documented | Corsaro et al. 1998<br>Molinaro et al. 2002<br>Zhang et al. 2003 |
| | Polymer of rhamnose, galactofuranose and mannose (unnamed) | Results in tissue necrosis through unknown mechanisms | Not documented | Corsaro et al. 1998<br>Molinaro et al. 2002 |
| | Galactan of galactofuranose residues (unnamed) | Results in tissue necrosis through unknown mechanisms | Not documented | Corsaro et al. 1998<br>Molinaro et al. 2002 |
| | Diaporthin | Interferes with protoplast permeability in *R. discolor*<br>Found in completely necrotized tissues far from the infection edge | Decreased, unchanged, or absent | Bazzigher 1953<br>Sparapano et al. 1989<br>Arnone et al. 2002 |
| | (+)-orthosporin | Results in tissue necrosis in some plant species, not tested with *Castanea* | Not documented | Sparapano et al. 1989<br>Arnone et al. 2002 |
| | Cryphonectric acid | Unique to *C. parasitica*, results in growth suppression in tomato seedlings | Not documented | Arnone et al. 2002<br>Teixeira et al. 2013 |
| | Emodin | Antibacterial | Unchanged | Sparapano et al. 1989 |
| | Skyrin | Orange pigment in mycelium<br>Interferes with protoplast permeability in *R. discolor*<br>Browning of inner bark in *Castanea* | Absent | McCarroll and Thor 1985a<br>Sparapano et al. 1989 |
| | Rugulosin | Responsible for yellow pigment<br>Necessary for sporulating mycelium<br>Found only in completely necrotized tissues far from the infection edge<br>Browning of inner bark in *Castanea* | Absent | McCarroll and Thor 1985a<br>Sparapano et al. 1989 |
| | Chrysophanol | Antibacterial | Absent | Sparapano et al. 1989 |

**Table 5.3.** Summary of factors influencing host defence.

| Constitutive factors in host defence contributing to more tolerant genotypes | References |
|---|---|
| • Thicker periderm with more layers of suberized phellem cells<br>• Additional periderm (rhytidome) forms quickly in response to infection<br>• Wound periderm formation begins ≤ 14 days post infection<br>• Thicker wound periderm, minimum of 9 phellem and 7 phelloderm cells<br>• Lesser concentration of hamamelitannin | Cook and Wilson 1915<br>Heberd et al. 1984<br>Griffin and Elkins 1986<br>Farias et al. 1995 |
| **Factors in host response upregulated by hypovirulence in *C. parasitica*** | **References** |
| • Greater chitinase expression in host<br>• Increased accumulation of salicyclic acid | Schafleitner and Wilhelm 1997<br>Schafleitner and Wilhelm 1998<br>Schafleitner et al. 1999 |

**Figure 5.1.** Mycelium of *C. parasitica* growing superficially (small arrows) and intracellularly (large arrows) in a *C. dentata* host (bar = 0.002 mm, stain = methylene blue).

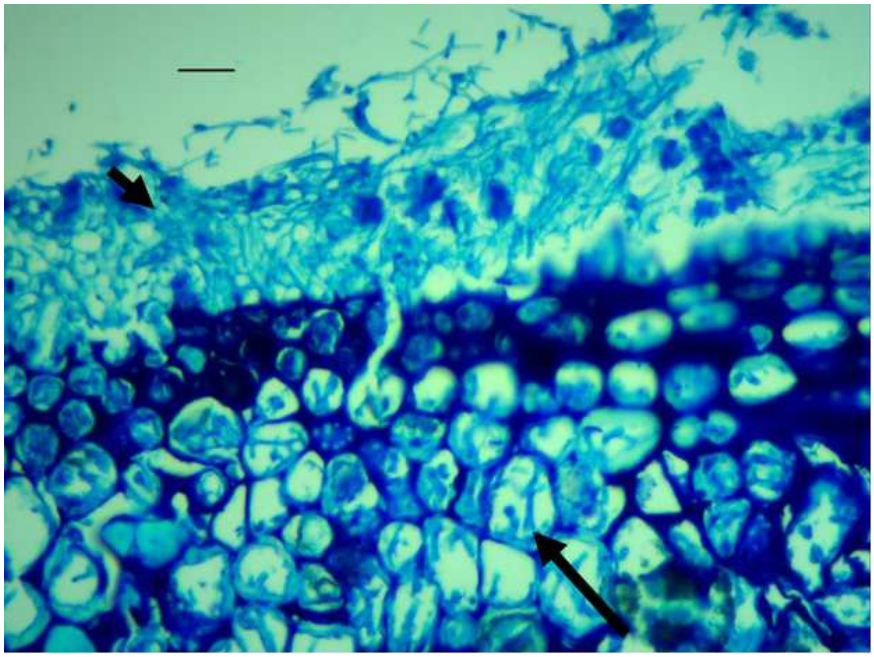


**Figure 5.2.** Advancing mycelia (small arrow), tannin deposition as a host defence response (tn), and wound periderm formation (large arrow) during active blight infection in *C. dentata* (bar = 0.3 mm, staining in safranin O and fast green).

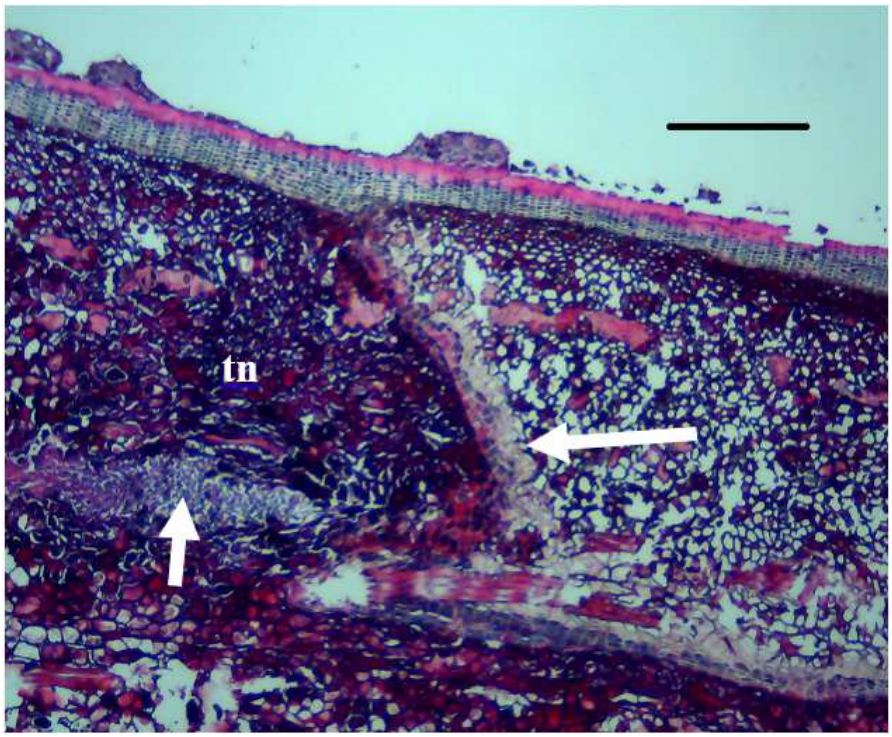


**Figure 5.3.** Relative periderm thickness and degree of suberization of the phellem layers may vary substantially between trees. A-C: three 2 yr old trees of *C. dentata*; periderm is similar in A and B, thicker and more suberized in C (bar = 0.3 mm, stained with safranin O and fast green).

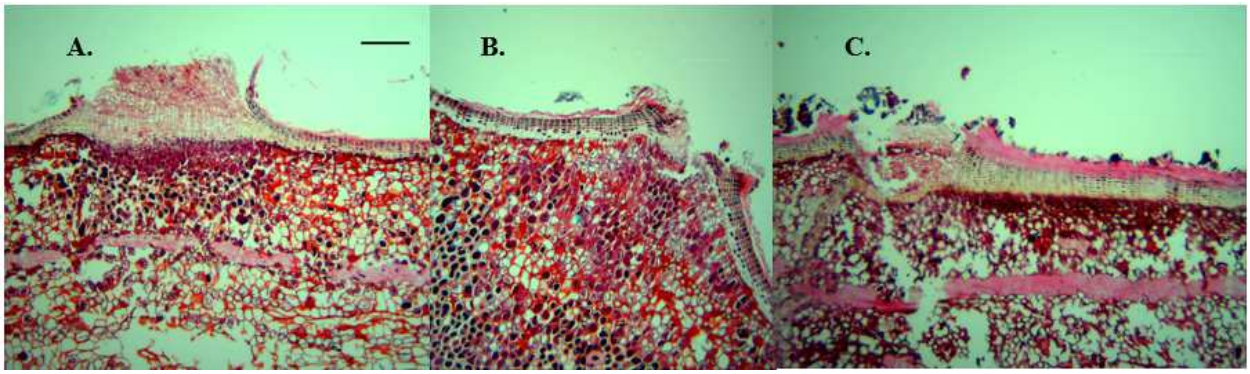
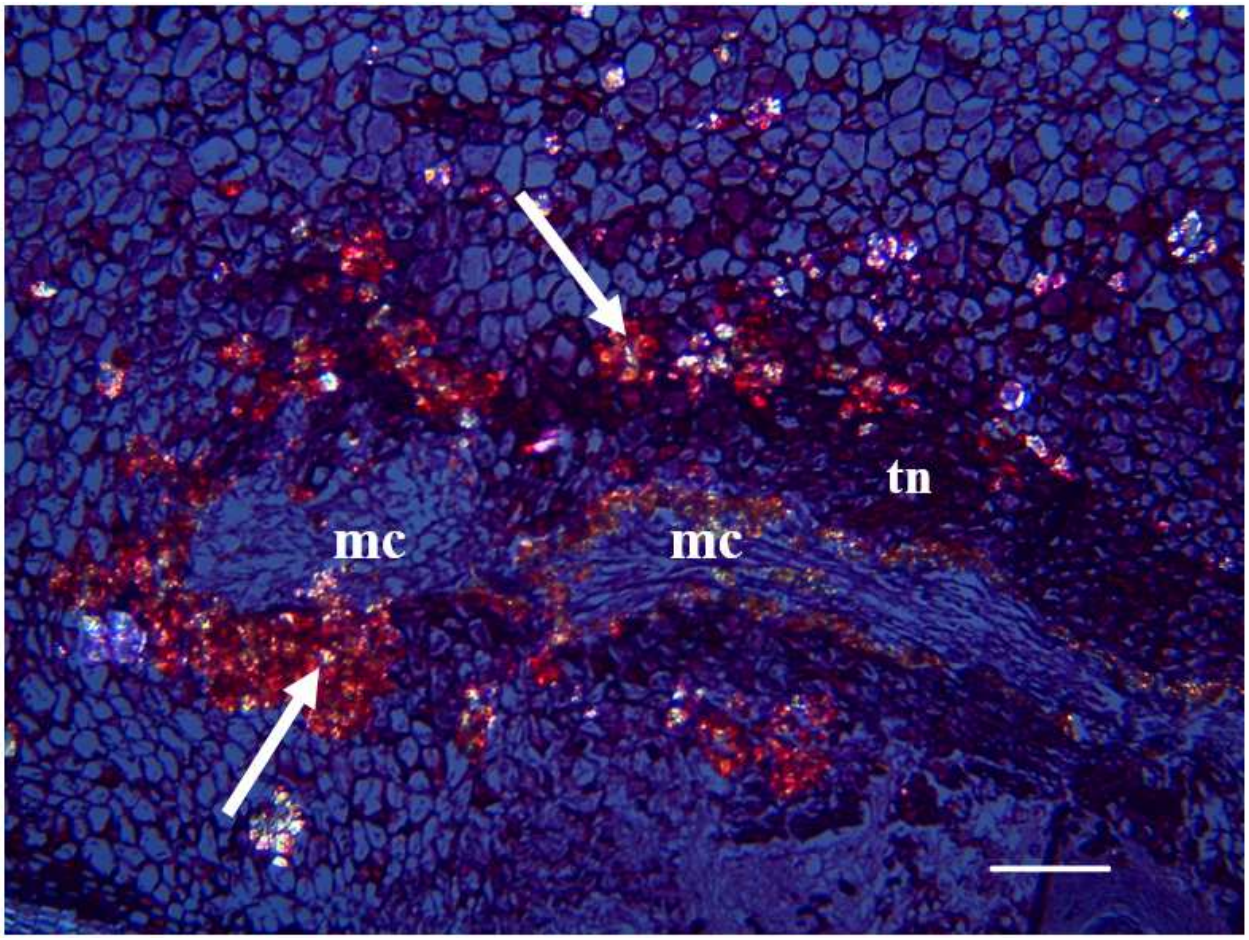



**Figure 5.4.** Phenotypic differences in culture between healthy and virulent (orange) *C. parasitica,* and a strain of *C. parasitica* infected with a hypovirus (white) (photo credit: Dr. Greg J Boland).

**Figure 5.5.** Appearance of calcium oxalate crystals (arrows) and tannins (tn) at the infection edge between advancing mycelium (mc) of *C. parasitica* and host tissues in *C. dentata* (bar = 0.01 mm, Stained with safranin O and fast green, viewed under a polarizing filter).



# CHAPTER 6. GENERAL SUMMARY AND CONCLUSIONS

## 6.1 General discussion

This thesis describes improved methods for axillary shoot culture and somatic embryogenesis for the purposes of short term conservation of the functionally extinct tree species *C. dentata* (Van Drunen et al. 2017). Factors such as growth-stage of the source material and incubation temperature, were investigated for their effect on multiplication rate in the axillary shoot culture of *C. dentata*. Axillary shoot culture multiplication rates were optimized at the genotype level for 22 different genotypes, and a conservation framework was recommended for continued work with this species. A mechanism of STN was also identified, and STN rates were reduced. Adventitious roots were produced from both leaf and stem tissues in culture. A somatic embryogenesis protocol was developed utilizing axillary culture microshoots as the source material, and with a significantly greater success rate in embryo induction over existing protocols utilizing immature zygotic embryos as explants (Merkle et al. 1991; Johnson et al. 2007). Of particular note in Chapters 3 and 4, are the observations made on the interaction between *C. dentata* and incubation temperature. Incubation temperature is rarely investigated in the literature (Corredoira et al. 2015). However, for some species temperature can have a dramatic effect on relative growth rate, and the results demonstrated in Chapters 3 and 4 suggest that this mechanism may be of particular importance in *C. dentata* (Lo Schiavo et al. 1990; Györgyey et al. 1991; Decout et al. 1994). In this thesis, documented metabolomics of the host-pathogen interaction between *C. parasitica* and *C. dentata*, were critically assessed; determining factors which should be targeted in any program for the generation of a blight-tolerant *C. dentata*. Findings in this thesis are outlined in greater detail below.

***Chapter 3***

The results outlined in Chapter 3 highlight the difficulties is developing an axillary shoot culture system for the purposes of conservation. The growth stage of the source material had a considerable effect on the overall multiplication rate, with cultures derived from seedling-stage material maintaining the greatest multiplication rate overall (7.87 ± 0.38). This generally agrees with observations in the literature, in which explants derived from juvenile-phase tissues are generally more vigorous than those from mature-phase tissues. However, embryo-stage genotypes are of a juvenile-phase as well, and in this study embryo-stage material had the lowest multiplication rate overall (2.95 ± 0.20). This may be a result of the process used to generate the

embryo-stage genotypes. The process of somatic embryogenesis may have left an epigenetic mark on these genotypes, resulting in relatively low vigor in axillary shoot cultures. In the axillary shoot culture of garland daphne (*Daphne cneorum*), selecting for specific growth habits (apically dominant or greater shoot proliferation) resulted in divergent phenotypes with measurable differences in responses to growth regulators and rooting success ex vitro (Marks and Myers 1994). Apical dominance is determined by the ratio of cytokinin to auxin in the plant, with greater cytokinin resulting in a more apically dominant shoot (Su et al. 2011). In selecting for apically dominant growth or a proliferation or shoots, Marks and Myers (1994) were likely selecting for two different auxin/cytokinin profiles that then became epigenetically fixed in culture. The procedure for somatic embryogenesis in *C. dentata* may modify the auxin/cytokinin ratio in genotypes in such a way that the resulting auxin/cytokinin profile is suboptimal for axillary shoot culture. Alternatively, the current protocol for somatic embryogenesis may be indirectly selecting for individuals that are recalcitrant to axillary shoot culture. The current success rate for somatic embryo induction in explant material is very low in *C. dentata* (0.02 – 3.00 %; Merkle et al. 1991; Johnson et al. 2007). As a result, it may be that only a very narrow allelic profile of genotypes contain the endogenous auxin/cytokinin that responds to the existing protocol for somatic embryogenesis, and this allelic profile may be recalcitrant in the existing axillary shoot culture procedure. The identification of genetic mechanisms involved in somatic embryogenesis in *C. dentata* may help resolve this issue. However, to date the genome of *C. dentata* is poorly characterized. As well, the inclusion of a greater number of genotypes from the embryo-stage could help resolve this issue. This is something which will be possible with the protocol outlined in Chapter 4 for somatic embryogenesis.

Based on the results obtained concerning growth-stage and culture vigor, to increase the chance of maximizing relative vigor in culture, genotypes of *C. dentata* should be explanted to culture from seedling-stage sources. However, Mettler was the most vigorous line included in this study, and was derived from mature-phase tissues. Therefore, it may be that the genotype, rather than growth stage of the source material, has the greatest effect on the relative vigor in culture. It was not possible to sample multiple growth stages from the same individual in this study. With a self-incompatible species such as *C. dentata*, it would not be possible to compare material derived from the embryo-, seedling-, and mature-phase based on established tissue culture protocols for this species in the literature (Merkle et al. 1991). Explant harvesting for somatic embryogenesis in

existing protocols is destructive to the reproductive unit. However, in the somatic embryogenesis protocol described in Chapter 4, no reproductive unit is needed. Therefore, this may be possible in future studies (see section 6.3). Sampling of both seedling- and mature-stage tissue from the same genotype is possible, if the study is initialized with seedling material and a sufficient period of time is allowed to pass for the source plant to develop into a mature tree. However, a study of this scope was not possible in the time frame of this work. To help control for the potential effect of genotype, many representative samples (genotypes) of each growth-stage were included. Somatic embryogenesis in *C. dentata* with existing protocols has a very low success rate, and as a result few genotypes were available in culture at the time of this study for comparison.

In this study, incubation temperature was shown to have a significant effect on multiplication rate in *C. dentata*. Several genotypes approached a commercial increase rate (approx. 10 X/6 wk) in CAM at 21 ± 1 °C (New 16, and New 17). Others, including Mettler and New 11, had an increased shoot multiplication rate under greater incubation temperature (31 ± 1 °C). Light Cemetery grew at twice the rate at 21 ± 1 °C compared with 31 ± 1 °C. The source plants for the genotypes included in this study were drawn from a similar isotherm (4 – 10 °C). This would suggest that all source material should have phenotypes adapted to local temperature conditions, and should respond similarly in culture. This is not what was observed in this study, where growth rate at the two incubation temperatures varied for some genotypes. It has been noted in other plant species that the preferred temperature of incubation in culture does not necessarily match the temperature optima of the source plant (de Capite 1955; Kvaalen and Johnsen 2008). In *Picea abies*, this phenomenon was related to the nature of temperature adaptation during zygotic embryo development (Kvaalen and Johnsen 2008). Individuals of *P. abies* acquire a determined temperature optimum based on the external temperature experienced as a zygotic embryo. This mechanism is believed to also be functioning during somatic embryogenesis, allowing for the programming of temperature preferences in the in vitro plantlet by manipulating the incubation temperature during somatic embryogenesis. Results from this work suggest that a similarly complex mechanism may be impacting incubation temperature optima in *C. dentata*. Identifying the best incubation temperature for a range of genotypes is challenging, but can be an efficient method for increasing the multiplication rate. None of the other 14 genotypes included in this thesis were tested empirically for the effect of temperature on multiplication rate. However, given the prevalence of temperature preferences found in the genotypes examined (three out of eight), likely

differences would have been found in a portion of the untested genotypes. This study has demonstrated the importance of determining the temperature at which growth rate is stimulated for each genotype of *C. dentata*.

For *C. dentata*, it is clear that there exists considerable range between the multiplication rates for individual genotypes in culture (ranging from 1.45 X/12 wk to 13.25 X/6 wk; Figure 3.3). For conservation, maintaining high genetic diversity in the genotypes preserved in culture is desirable (Corredoira et al. 2017). It is inevitable that in the pursuit of maintaining many diverse genotypes in culture, a single standard protocol is unlikely to accommodate all, or even the majority, of specimens within a species. It is likely that modifications will have to be made to existing protocols in order to accommodate genotypes recalcitrant under the standard protocol for the species. In the results described in Chapter 3, three variations on a standard axillary shoot culture procedure were used to increase multiplication rates across the 22 genotypes investigated in this study. For the majority of genotypes (18 out of 22), cultures could be maintained on CAM under 21 ± 1 °C over a 6 wk subculture schedule. Two genotypes (664 and Zrite) grew too slowly and needed a modified 12 wk subculture schedule, still on CAM and under 21 ± 1 °C. Two genotypes (Mettler and New 11) were more proliferative when incubated at a greater temperature (31 ± 1 °C), on CAM and with a 6 wk subculture schedule. These differences in subculture schedule and incubation temperature can greatly affect the multiplication rate. This variation should be taken into account when propagating other genotypes of this species in culture.

STN occurred in all genotypes included in this study. However, modifications to exogenous medium components failed to alleviate rates of STN in any genotype without also supressing growth rates, with the exception of 4.0 µM BAP. At the greatest exogenous concentration of BAP (4.0 µM), % STN was significantly reduced relative to the control, while there was no statistically significant difference between plant height, microshoot production rate, or the overall multiplication rate. In the closely related *C. sativa*, the direct application of 5.0 µM BAP to the apical tip was observed to reduce the incidence of STN from 76.7 % to 0.0 % (Piagnani et al. 1996). This would suggest that increasing exogenous BAP beyond the 4.0 µM in the medium examined in this study could reduce levels of STN even further. However, increasing cytokinin concentration has been noted to decrease plant height and multiplication rate in some plant species (Alosaimi et al. 2018). As well, response to increased cytokinin is species specific, and can even be specific to the particular cytokinin. In the axillary shoot culture of Garett's hummingbird

trumpet (*Epilobium canum* subsp. *garrettii*), the cytokinins BAP and K did not affect plant height between treatments at levels 0.0, 1.1, 2.2, 4.4, and 8.8 µM. However, shoot height was significantly reduced at TDZ treatment of 4.4 and 8.8 µM, relative to treatments of 0.0, 1.1, and 2.2 µM. Therefore, it should not be assumed that increasing the exogenous concentration of BAP above 4.0 µM could eliminate % STN in *C. dentata*. As well, increasing cytokinin levels in the medium has been found to reduce rooting rates in the closely related *C. sativa* (Vieitez et al. 1989). The microshoots produced in the BAP trial of this study were not tested for rooting rates in Stage IV. However, given the observations of Vieitez et al. (1989), rooting rates in microshoots cultured with increased exogenous BAP in the medium will have to be tested for reduction in rooting capability before the implementation of increased BAP in the medium of *C. dentata* as a method to reduce % STN.

The most concentrated treatments of exogenous Ca (6.0 mM) supressed shoot height, microshoot production rate, and multiplication rate relative to the control. This suggests that at 6.0 mM of added calcium, osmotic potential in the medium is increased to the point where it is interfering with transpiration rate, supressing growth. Studies with *C. sativa* have applied external Ca to the apical tip of microshoots, both during Stage II and during Stage IV rooting (Piagnani et al. 1996). Adding as little as 0.39 ± 0.02 nM Ca, complexed in agar, reduced the incidence of STN to 30.0 % relative to the controls (of 76.7 %). Results with *C. sativa*, and the reduced growth resulting from the 6.0 mM Ca treatments in this study, suggest that although Ca deficiency at the apical tip is related to the incidence of STN, it is the translocation of Ca from the medium to the apical tip that remains the issue. Alternatively, at 6.0 mM exogenous Ca, *C. dentata* may be exposed to toxic levels of Ca (Macnicol and Beckett 1985). This may be independent of potential increases in osmotic potential in the medium, or both effects may be interacting. Added B at the levels investigated in this study did not differ from the control. However, the addition of 0.025 mM B to the 6.0 mM treatment did result in plant height, microshoot production rate, and multiplication rate in the same range as the control, suggesting that at the levels tested in this study, B does help facilitate the uptake of Ca, as was observed with *S. tuberosum* (Abdulnour et al. 2000). However, cultures in the Ca/B 6.0/0.025 mM this treatment did not perform better than the control, either in growth rate or in reducing % STN.

At the treatment levels and with the gelling agents investigated in this study, STN rates did not differ between treatments. Plant height, microshoot production rate, multiplication rate, and %

STN were similar across all gellan gum treatments and the 6.0 g/L agar treatment. Shoots in the 7.0 and 8.0 g/L agar treatments had significantly lesser height, microshoot production rate, and overall multiplication rate, with no difference in % STN. At the treatment levels of gellan gum and agar examined in this study, studies have measured osmolality and water potential to be similar across treatments and gellan gum and agar similar, ranging from 230.0 ± 50.0 to 240.0 ± 20.0 mOsm/kg and - 11.3 ± 2.7 to -11.6 ± 1.1 Bar respectively (in full strength MS and 30.0 g/L sucrose; Scherer et al. 1988). However, gellan gum and agar can contain very different concentrations of key micronutrients, in particular B, Ca, and Mg. Gellan gum contains 4900.0 µg/g Ca relative to 110.0 µg/g in agar, and 1530.0 µg/g of Mg relative to 62.0 µg/g in agar. Agar contains 23.0 µg/g of B, while gellan gum contains only 1.4 µg/g. It may be that at the agar concentrations of 7.0 and 8.0 g/L, concentrations of specific micronutrients, such as B, may be reaching concentrations toxic to growth (Debergh 1983; Macnicol and Beckett 1985). To examine this in detail, micronutrients found in greater concentration in agar would need to be isolated and examined individually, or in concert with potentially interactive additional micronutrients, such as the co-transport possible between Ca and B (Abdulnour et al. 2000). Alternatively, at higher concentrations of gelling agent, cytokinin may become increasing unavailable (Debergh 1983). Increasing gellan gum concentration (from 1.3 g/L to 5.0 g/L) in the axillary shoot culture of the small-flowered daisy-bush (*Olearia microdisca*) microcuttings, reduced the effect of a 20.0 µM treatment of BAP (Williams and Taji 1991). In the formation of adventitious buds of *P. abies*, Bornman and Vogelman noted an inverse relationship between gel rigidity and uptake of BAP by plant tissues in both agar (1.5 – 15.0 g/L) and gellan gum (1.0 – 5.0 g/L). They even suggest that the maximum availability of BAP may only be possible in the complete absence of a gelling agent. Many different mechanisms, alone or with an interactive effect, may be involved in the effect of gelling agent compound and concentration on *C. dentata*. Unfortunately, none of the treatment levels investigated in this study has a significant effect in either reducing rates of STN or increasing multiplication rates.

Results described in Chapter 3 allow for the development of a genotype-optimized axillary shoot culture system which improves both multiplication rate and plantlet quality (through potentially reduced % STN) over existing protocols (Keys and Cech 1982; Serres et al. 1990; Xing et al. 1997; Yang et al. 2009). The residual effect of 4.0 µM BAP in the axillary shoot multiplication medium on a later stage (Stage IV) ex vitro rooting rates will need to be investigated

before this increased concentration of BAP can be recommended for inclusion in the axillary shoot culture protocol.

***Chapter 4***

Existing protocols for the somatic embryogenesis of *C. dentata* could not generate somatic embryos from leaf or stem tissues (Merkle et al. 1991; Corredoira et al. 2015). Instead, only immature zygotic embryos harvested 6 – 7 wk post-anthesis could be used to produce somatic embryo cultures, and these had a low induction rate of 0.02 – 3.00 % (Merkle et al. 1991; Carraway and Merkle 1997; Xing et al. 1999; Johnson et al. 2007; Merkle et al. 2011). Chapter 4 describes multiple treatment strategies that were used to produce somatic embryos from vegetative tissues derived from axillary shoot culture. Specifically, a series of NAA/BAP, TDZ, and 2,4-D/BAP treatment strategies were used. It was observed that specific ratios of NAA to BAP (5.0/1.0 µM and 10.0/1.0 µM) would produce adventitious roots from internodal stem sections and leaf cuttings. Adventitious root tissues are difficult to produce from *C. dentata* in culture, therefore the production of these organs are noteworthy (Xing et al. 1997; Oakes et al. 2016). TDZ treatments at any level investigated in this study failed to stimulate the production of somatic embryos. TDZ treatments have proven useful in inducing somatic embryogenesis in some woody plant species previously recalcitrant to somatic embryogenesis. However, species-specific responses to growth regulators can vary widely. In this study, when TDZ induced callus formation in microcuttings of *C. dentata*, callus quality was similar across treatments and mainly differed across genotypes. Although some of the callus produced (creamy and nodular) was visually similar to the callus which produced adventitious roots with NAA/BAP (10.0/1.0 and 5.0/1.0 µM), no adventitious organs were produced. At the treatments of TDZ tested in this study and the genotypes examined, TDZ alone was not sufficient to induce somatic embryogenesis. The production of somatic embryos occurred with the 1.0/1.0 µM 2,4-D/BAP treatment regime, improving somatic embryogenesis induction rates to 14 – 19 (out of 20 microcuttings) for two out of the three genotypes tested. Microcutting type was found to be instrumental in the production of somatic embryos, requiring a single node cutting as opposed to isolated microcuttings of internodal stem sections or leaf cuttings. Likely the particular auxin/cytokinin present in single node cuttings facilitated the response of this microcutting type to the 2,4-D/BAP treatment (Su et al. 2011). Leaf or internodal stem microcuttings likely had different profiles of endogenous growth regulators, and these profiles were not responsive to the 2,4-D/BAP treatment tested in this study.

Incubation temperature and continuous light may have played a role in somatic embryo formation. In Chapter 3, it was demonstrated that temperature affected the shoot growth in some genotypes of *C. dentata*. It may be that temperature also affected somatic embryogenic potential in *C. dentata*. Temperature is rarely studied in somatic embryogenesis (de Capite 1955; Watts et al. 1984; ten Hoopen et al. 2002). However, there is work supporting the potential role of temperature in somatic embryo formation. For example, in the somatic embryogenesis of a *Cichorium* hybrid [*Cichorium intybus* L. x *Cichorium andivia* L.), temperature was integral in stimulating somatic embryogenesis (Decout et al. 1994). On a single medium, if leaf cuttings of this hybrid were cultured at 20 – 25 °C, callus and shoot formation occurred. If cultured at 35 °C, direct somatic embryogenesis occurred. At the intermediary temperature of 30 °C, all forms of morphogenesis were observed. It is possible that the activation of heat shock proteins (HSP) by high temperature incubation conditions may be facilitating somatic embryogenesis. A relationship between HSP and somatic embryogenesis has been noted during somatic embryogenesis in *Medicago sativa* (Györgyey et al. 1991). In the early stages of somatic embryogenesis (to the globular stage), HSP expression was increased 7×. Expression gradually declined as embryos developed, until by the torpedo stage of development HSP expression was at a level comparable to before the initiation of somatic embryogenesis. In carrot, a mutant (ts1cc) has been generated which can be arrested by changes in incubation temperature at particular stages of development during somatic embryogenesis (Lo Schiavo et al. 1990). In this instance, it is the high temperatures which supress embryo development. Incubating ts1cc at 24 °C will allow somatic embryogenesis to proceed through to Stage IV. However, incubation at 32 °C will arrest the development of somatic embryos at the globular stage, despite any modifications to the medium. In this model, HSP are not responsible for arresting development. Instead, temperature modified the glycoproteins synthesized by the mutant, significantly reducing the frequency of fucose in the glycoproteins, and as a result they do not accumulate at the periphery of the embryo (likely contributing to polarity) but instead diffuse equally throughout the embryo. This study only examined a single temperature (31 ± 1 °C) in the somatic embryogenesis of *C. dentata*, and with two genotypes (AcGraft and ASH1) which were not included in the temperature trial in Chapter 3. From the results obtained in this thesis, it is clear that incubation temperature affects growth in at least some genotypes of *C. dentata*. More work needs to be done to investigate the range of temperature affects and genotype specificity of this interaction. Despite the work which remains

to be done, the development of a somatic embryogenesis protocol utilizing axillary shoot cultures as source material will improve the utility of somatic embryogenesis in the short term conservation of *C. dentata*.

***Chapter 5***

In Chapter 5, existing metabolomic literature is compiled and assessed for the host-pathogen interaction between *C. parasitica* and *Castanea* spp.. In this work, over 100 yr of research is collected and utilized to rank and classify potential virulence factors based on existing literature, and to identify possible host defence factors, both constitutive and expressed. Important virulence factors to be targeted in engineering blight tolerance in the host include: oxalate, laccase, polygalacturonase, and tannase. Other compounds included in the review may be important to the virulence of *C. parasitica*, but more work needs to be done to elucidate their true role during an active blight infection. An alternative approach would be to increase existing host defence mechanisms, such as increasing the speed of pathogen recognition or wound periderm formation. The efficient production of somatic embryos, described in Chapter 4, will greatly facilitate breeding efforts through genetic engineering. The review outlined in Chapter 5 will allow for the identification of relevant virulence factors and host-defence factors for targeting in a transformation-based breeding program, in order to improve blight tolerance in *C. dentata* (Merkle et al. 2007). Such a breeding program has already produced a limited number of lines with increased blight tolerance through the expression of an oxalate oxidase gene, which are currently awaiting approval for public release (Zhang et al. 2013). However, single gene pathogen resistance is often overcome by adaptation in the pathogen, and it would be beneficial to introduce a greater number of blight tolerance genes (Moury et al. 1997). As well, the limited success in previous somatic embryogenesis protocols has resulted in a very limited number of transformed lines, resulting in a genetic bottleneck in the breeding program (Merkle et al. 1991). Chapter 5 also contains only the second histological images of an active blight infection in *C. dentata* over the past 100 yr, and the first such examination of juvenile-phase host tissues. These images both support the existing publication (Hebard et al. 1984), and expand on this work by documenting structures (such as oxalate crystals at the periphery of an active mycelial fan, the relative periderm thickness of putatively susceptible and tolerant *Castanea*, and tannin expression) which, to the knowledge of the authors, have not been published before.

Through the combined work detailed in Chapters 3, 4, and 5, significant improvements have been made to existing short term tissue culture conservation programs with *C. dentata*. Section 6.4 describes how this work will be directly utilized by the TACF and CCC within the next year in order to improve their existing *C. dentata* conservation programs.

## 6.2. Strengths and limitations

A great strength of this study is the large number of genotypes incorporated into this work. For a threatened species, it is very difficult to assemble a large group of genotypes for study, particularly from diverse geographic locations and distinct sub-populations (Husband 2016; Gailing and Nelson 2017; Van Drunen et al. 2017). The inclusion of this diverse genetic base has enabled the development of methodologies and recommendations for culturing this species both on the species level, and at the much finer level of the genotype (Husband 2016; Gailing and Nelson 2017). Progress described in this thesis was accelerated by contacts from within the diverse network of researchers studying *C. dentata* throughout North America. The contribution of genotypes to this study by diverse groups of researchers was previously described in Chapter 3. Mentorship and advice provided by this community also contributed to the outcomes described. Specific individuals are mentioned in the Acknowledgements.

There are several limitations to the experimental designs included in this thesis. The most concerning is a lack of genotypes from the more southern parts of the natural range of *C. dentata*. The author did not have connections within the southern chapters of the TACF, and thus did not acquire nuts from these sub-populations. It is possible that these southern sub-populations would respond very differently to some of the factors measured in this study, in particular abiotic factors such as temperature and light level. Both of these are likely to be experienced differently by southern sub-populations compared to the more northerly genotypes included in this study (Lovat 2013). Recent genetic work has determined that *C. dentata* populations in southern Ontario are most closely related genetically to sub-populations from the southwestern portion of the *C. dentata* range, and were likely introduced to Canada through human-mediated dispersal events (Gailing and Nelson 2017). Therefore, it is not clear if southwestern populations would be very distinct phenotypically from existing Canadian genotypes. Another limitation in the source material is the low number of embryo-stage genotypes included in this study. This sample may or may not be representative of genotypes derived from embryo-stage tissues. Another limitation to this research was a result of the facilities available during this study. It would have been greatly beneficial to

the interpretations of the results of Chapter 3 and 4, if more than two different temperature regimes could have been examined. Within the scope of this thesis, only two temperatures were examined as a result of a limited availability in growth chamber space. A minimum of three would have allowed for an initial investigation of a relationship between growth rate and temperature (see Section 6.3). A further limitation was imposed by time. The production of somatic embryos was discovered very recently; towards the end of the research interval. As a result, there was not sufficient time to test whether somatic embryos could undergo germination. However, this deficiency will be addressed by the researchers at SUNY-ESF in the coming years (see section 6.4).

A limitation across all aspects of this study includes deficiencies in the understanding of the genome of *C. dentata*. Although not a feature investigated directly in this thesis, a lack of knowledge concerning the *C. dentata* genome and regulatory mechanisms prevents the gene-level examination of the tissue culture methodologies developed in this thesis. In particular, it would be very interesting to determine which mechanisms may be involved in temperature response during somatic embryogenesis, as well as potential differences in the regulatory mechanisms involved in somatic embryogenesis from immature zygotic tissues (Merkle et al.'s 1991 protocol) and the somatic embryogenesis induced from axillary shoot cultures outlined in Chapter 4. With the ongoing genomic work of the Hardwood Genomics Project, it is hoped that this genomic information will eventually be available for use (Carlson et al. 2014).

### 6.3. Considerations for future research

Several avenues of research emerge from the results published in this thesis. Although the results from Chapter 3 clearly indicate that explants derived from 4 mo old seedlings generally produced the most vigorous cultures, it was not possible to compare the relative vigor of different growth stages derived from the same genotype. It would be beneficial to compare the relative vigor rates of seedlings, mature-phase grafts, and germinated somatic embryos derived from the same genotype. For this purpose, somatic embryos could not be harvested as recommended in the older literature (Merkle et al. 1991). However, with the somatic embryogenesis methodology developed in Chapter 4, a genotype could be entered into axillary shoot culture during the seedling-stage, and then utilized to produce somatic embryos for germination and comparison as embryo-stage tissues. It may even be possible to also produce somatic embryos from the cultures derived from the mature-stage tissues of this same genotype, potentially producing four levels of tissue growth stage

to compare per genotype (seedling-, mature-, seedling/embryo-, and mature/embryo-stage). This would allow for a more accurate determination of the relative vigor of material of different growth stages, independent of the confounding factor of genotype. Assays could also be conducted to compare the relative ratios of endogenous growth regulators among these four groups, providing a possible explanatory mechanisms for differences in relative vigor in culture (Farrow and Emery 2012; Liu et al. 2012). However, this would require both seedling material, and the time to allow field source plants to reach maturity. As well, this work would require a large number of genotypes, as it is not yet clear how many genotypes are competent in the somatic embryogenesis protocol outlined in Chapter 4. Despite these limitations, this would be a realistic study.

From the work presented in Chapter 3, it is suggested that temperature preference should be investigated in a wider range of genotypes, particularly with genotypes from the southern range edge of *C. dentata*. It would be preferable to conduct a climate gradient phenology study, sampling sub-populations from specific isotherms and testing their growth response to temperature in culture (Lovat 2013). It would also be beneficial to expand the range of temperatures examined, including a central temperature of 25 °C and a more extreme cold temperature of 15 °C (Kristiansen and Andersen 1993). This would allow for the creation of a temperature gradient to track against growth, facilitating the determination of a temperature optima for growth across genotypes (Kvaalen and Johnsen 2007). As well, elaborating further on the work with STN conducted with *C. dentata*, it would be useful to repeat the experimentation of Piagnani et al. (1996), conducted with localized tip applications of BAP or Ca to the apical shoot tips of *C. dentata* in order to demonstrate that these factors are conclusively missing from the apical tip during STN. This study should also include a Stage IV rooting component, in order to determine if the BAP or Ca treatments would adversely affect rooting as was documented with *C. sativa* (Vieitez et al. 1989). A successful axillary shoot culture procedure must include a successful Stage IV rooting and acclimatization stage (see Chapter 8). Any treatment which alleviates STN, but reduces rooting, cannot be implemented.

With the results obtained in Chapter 4, as with Chapter 3, it would be of interest to continue investigating the role of temperature in the somatic embryogenesis of *C. dentata*. The incorporation of a temperature gradient, ranging from 15, 20, 25, and 30 °C, would demonstrate how impactful temperature is on the induction of somatic embryogenesis. Unfortunately the genetic mechanisms involved in heat stress tolerance in *C. dentata* have yet to be determined. As

such, it is currently not possible to determine if changes in expression of HSPs may be inducing somatic embryogenesis. However, this could be an avenue of research pursued with the ongoing genomic work with the Hardwood Genomics Project (Carlson et al. 2014). As a part of this project, the genome of *C. dentata* is being compiled over time by multiple researchers. Once completed, regions associated with HSP in *C. dentata* could be identified, and tracked for expression during somatic embryogenesis (Györgyey et al. 1991). As well, it is of great interest to determine if genotypes derived from mature-phase tissues can be induced to form somatic embryos. The Mettler genotype examined in Chapter 4 proved to be phenotypically unrepresentative (more vigorous) of the majority of the genotypes derived from mature-phase tissues. Therefore, it would be of considerable interest to repeat this study with a greater range of mature-phase genotypes, as this may facilitate the inclusion of phenotypically described mature trees into current transformation programs. As well, work needs to be conducted to determine that the somatic embryos generated from the 1.0/1.0 µM 2,4-D and BAP treatments can be germinated into full plantlets. In the results reported in Chapter 4, embryos reached the heart stage, and possibly early torpedo stage, before senescence. In the germination of somatic embryos from immature zygotic tissues, generally a hormone-free regeneration medium, a high-cytokinin medium (essentially the Stage II axillary shoot medium) or cold treatment are utilized to facilitate the germination of embryos (Carraway and Merkle 1997; Xing et al. 1999; Johnson et al. 2007). Embryo germination has not traditionally been a limitation in studies utilizing explant tissues of immature zygotic embryos, reporting relative high success rates for somatic embryo germination (9.9 % – 80.0 %) relative to the somatic embryo induction success rate (0.02 – 3.00 %). However, given the different source material and growth regulator concentration utilized in this study, it is possible that different transcription factors have been activated in the microcuttings utilized in Chapter 4 compared to previous studies (Smertenko and Bozhkov 2014). Therefore it is possible that methods successful with existing protocols will not be successful with the somatic embryos generated through the protocol outlined in Chapter 4. Work needs to be done to confirm the successful germination of embryos derived from 1.0/1.0 µM 2,4-D and BAP.

Finally, in vivo metabolomics work needs to be conducted on cankers during active blight infection to test for the presence of virulence factors outlined in the review of Chapter 5. If any compounds can be demonstrated as active in the leading edge of the canker, then the targeting of these compounds with genes producing the appropriate pathogenesis-related protein would be

highly beneficial in the development of blight-tolerant *C. dentata*. This mechanism has already been demonstrated with oxalate oxidase in *C. dentata* (Zhang et al. 2013). However, single gene resistance is often rapidly overcome by pathogens. Therefore it would be useful to stack multiple resistance genes coding for different pathogenesis-related proteins into *C. dentata*, as this helps reduce the probability that chance mutations will allow a pathogen to completely overcome the host defences.

The work presented in this thesis solves some important issues in the tissue culture of *C. dentata*, particularly for conservation purposes. However, it also raises many important avenues for further research with this species. Many promising methodologies are now available to aid in the conservation of *C. dentata* into the 21st century.

## 6.4. Conclusions

In summary, the work presented in this dissertation represents significant developments in the short term conservation of *C. dentata* through tissue culture. A framework was developed for an axillary shoot culture protocol which could be applied to a genetically diverse range of genotypes of *C. dentata* (Husband 2016; Gailing and Nelson 2017). A methodology was developed which allows for the production of somatic embryos from vegetative tissues, and success rates for somatic embryogenesis were greatly improved. Finally, the mechanisms involved in the host-pathogen interaction between *C. parasitica* and *Castanea* were identified from the literature. The results obtained from this dissertation are of immediate use to the conservation of *C. dentata* by organizations both in the United States and Canada. The SUNY-ESF research group has received the genotypes cultured in this thesis, and will be refining and expanding upon the somatic embryogenesis procedure published here in order to increase the number of distinct genotypes transformed and entered into their genetic engineering program (Zhang et al. 2013). The review presented here will be used to help them expand their existing roster of targeted virulence factors beyond oxalate. As well, the CCC will be incorporating the axillary shoot production into their "Breaking Isolation" program detailed in Chapter 3 in order to produce large numbers of existing select trees of *C. dentata* in the remaining Canadian sub-population. This is a large component of their provincial recovery plan, and will be responsible for generating around one third of the trees they will be planting (approx. 6000 trees will be planted over the next 2 yr, in existing mixed forests and as seed plantations). Work with the SUNY-ESF group has demonstrated that trees derived from axillary shoot culture are phenotypically similar to trees produced through sexual

reproduction (Newhouse et al. 2014). Great success has been found in implementing these short term conservation methodologies with *C. dentata*. It is hoped that future work will expand on the concepts investigated here with *C. dentata*, and this framework applied to the preservation of other imperiled plant species.

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# CHAPTER 8. APPENDIX

## 8.1 Methods for ex vitro rooting and acclimatization of American chestnut (*Castanea dentata* (Marsh.) Borkh.) microshoots

### 8.1.1 Introduction

In this thesis, protocols were described for the use of tissue culture in the short term conservation of *C. dentata*, including: Stage 0 (Chapter 3), improved Stage II protocols for axillary shoot culture (Chapter 3), and a Stage II protocol for somatic embryogenesis from axillary shoot tissues (Chapter 4). Maintenance and proliferation of *C. dentata* through axillary shoot culture is a central feature of this thesis. However, axillary shoot culture must end with a satisfactory Stage IV. Stage IV for *C. dentata* is difficult, with high losses noted (Xing et al. 1997; Oakes et al. 2013; Oakes et al. 2016). Rooting protocols reportedly lead to successful rooting (from 10.0 – 93.0 %). However, published rooting protocols have been investigated with only one (Oakes et al. 2016) or two genotypes (Xing et al. 1997; Oakes et al. 2013) of *C. dentata*. Rooting success is highly genotype specific in some species (Vieitez et al. 2007). Therefore, a protocol tested on only a single or two genotypes may not be as successful or useful when applied to a wider range of genotypes. As well, survival of rooted plantlets through acclimatization was unreported, or poor (Xing et al. 1997; Oakes et al. 2013; Oakes et al. 2016). For example, Oakes et al. (2016) reported a rooting rate of 93.0 %. However, only 17.9 % - 23.4 % of rooted plantlets survived acclimatization to lower humidity. Losses were mainly through desiccation.

This section describes a Stage IV rooting and acclimatization protocol developed for use following the axillary shoot protocol outlined in Chapter 3 (Figure A.1). Success rates for rooting varied widely across genotypes, with some genotypes recalcitrant to rooting under the current protocol (New 17), while others had rooting rates of > 70.0 % (AcGraft). Once roots were established, acclimatization occurred successfully, independent of genotype (> 90.0 %). Although biological replication occurred (three genotypes), time precluded adequate statistical replication. Still, this work represents the first and only full acclimatization procedure (from Stage II to field transfer) available for *C. dentata*, and reports survival rates well above those described in the literature.

### 8.1.2 Protocol development

***Plant material***

All material for Stage IV was derived from microshoots cultured on CAM. Microshoots were maintained at 21 ± 1 °C at 60 µmol $m^{-2}s^{-1}$ for 16/8 h day/night cycle and subcultured every 6 wk. For Stage IV, microshoots were harvested at between 6 – 7 wk of age. Microshoot height is important to rooting success in *C. dentata* (Oakes et al. 2016). For this reason, only microshoots ≥ 3.5 cm were used. This allowed sufficient stem length (approx. 1.5 cm) for planting into the rooting medium, while a portion of the shoot (approx. 2.0 cm) remained above ground.

Microshoots were cut from the vascularized callus ball. The basal portion of the stem was cut at a 45 ° angle to increase the surface area of the stem exposed to rooting hormone. Microshoots were handled gently to ensure that delicate leaves were not broken or dislodged from the stem. In preliminary work, it was observed that leaf loss contributed to transplant failure.

*Rooting ex vitro*

In previous reports on *C. dentata* Stage IV, microshoots were rooted prior to acclimatization (Xing et al. 1997; Oakes et al. 2013; Oakes et al. 2016). Preliminary work in the development of this protocol confirmed that microshoots could not acclimatize to lower RH or greater light unless first rooted.

Two different IBA treatments (powder, liquid) at three different concentrations each were tested for their effect on rooting in *C. dentata* microshoots: (1) the rooting hormone powder Stim-Root (Plant-Prod, Brampton, Ontario, Canada) was tested at 0.1 %, 0.5 %, and 0.8 % active ingredient (IBA). (2) A liquid solution of IBA (Sigma Corporation, New Jersey, USA) was mixed at a concentration of 1.0 %, 2.0 %, and 4.0 %. IBA was dissolved in 0.5 mL of 1 M NaOH then brought to volume with double distilled water. For the Stim-Root treatment, microshoots were dipped into the powder up to the soil line (approx. 1.5 cm) before planting. For the liquid IBA treatments, microshoots were dipped into the IBA solution to approx. 1.5 cm, and held in the solution for 1 s.

Treated microshoots were planted into a peat based horticultural potting mixture (G6, Farfard, Quebec, Canada) and kept under 100 µmol $m^{-2}s^{-1}$ cool white fluorescent light at 21 ± 1 °C in an incubator. The potting mixture was fertilized with half strength 30-10-10 fertilizer (Plant-Prod, Brampton, Ontario, Canada). Microshoots were planted into a covered seedling tray, 10 in a row and 100 per tray. The tray was sealed completely with Parafilm to maintain a high RH (± 5.0

% of the RH measured in the Magenta© GA7 container). RH measured in the sealed GA7 vessels with microshoots was approx. 69.0 – 73.0 % as measured with a hygrometer probe.

For each trial, a minimum of 10 microshoots were utilized per genotype/treatment combination, and trials were replicated a minimum of three times over a 2 yr period. Full trials with replication were performed with AcGraft, ASH1, and New 17.

All IBA treatments induced root formation in *C. dentata* microshoots, and rooting rates were not significantly different between treatments across all three replicates. Rooting rates were highly genotype specific. For New 17, rooting rates ranged from 0 – 3 microshoots rooted (across all replicates and treatments). Rooting rates were greater in AcGraft and ASH1. In AcGraft, rooting ranged from 4 – 9 (across all replicates and treatments). Results with ASH1 ranged from 3 – 6 (across all replicates and treatments). Rooting treatments were found to be easier to conduct with rooting hormone powder, therefore all rooting was conducted with 0.8 % IBA Stim-Root after this trial.

Although not included as part of a controlled study, relative rooting rates were recorded for other genotypes included in Chapter 3 (Table A.1). These were classified into five groups based on rooting tendency and testing: responsive (majority rooting), variable (approx. 50.0 % rooting, results variable between replicates), recalcitrant (rooting possible, but generally < 30.0 %), unresponsive (rooting tested, but did not occur), and those not assessed.

***Acclimatization***

Preliminary work (Figure A.2) determined that acclimatization involved two discrete steps: (1) adjustment to lower RH, and (2) adjustment to increased light conditions. Both steps were affected by fertilization frequency.

**Acclimatization to lower RH**

In preliminary work it was seen that newly rooted shoots (plantlets) did not respond well to increased light. This may seem counterintuitive, as *C. dentata* in the wild grow opportunistically under increased light conditions (Joesting et al. 2009). However, increasing light more than 4 X the in vitro light levels (from 60 µmol $m^{-2}s^{-1}$ to > 300 µmol $m^{-2}s^{-1}$) caused light stress that generally resulted in leaf damage or plantlet death (Figure A.3). For this reason, newly rooted plantlets were first acclimatized to lower RH.

In vitro microcuttings and newly rooted plantlets of *C. dentata* also showed very low tolerance to decreased RH. Any exposure of leaf or stem tissues formed in vitro to ≤ 50.0 % RH

resulted in wilting beyond the permanent wilting point after 20 min. However, *C. dentata* plantlets were found to naturally become acclimatized to ≤ 50.0 % RH over time through the production of new tissues ex vitro.

After rooting, plantlets began producing flushes of new growth. During culture, leaves of *C. dentata* plantlets ranged in size between approx. 0.3 - 3.0 cm. Each new leaf flush in the rooted plantlet resulted in larger sized leaves relative to the previous flush (Figure A.4). It was determined through trial and error that when the new leaves had reached approx. 10.0 cm in size (3 - 5 leaf flushes after rooting), plantlets could be removed from high RH conditions in covered containers (69.0 – 73.0 ± 5.0 %) and grown at approx. 50.0 % RH in a growth chamber. Leaves produced in vitro desiccated when plantlets were moved to ≤ 50.0 % RH, but the leaves formed ex vitro persisted. The period of time required to produce leaves ex vitro was between 4 – 6 wk. This process was not found to be genotype dependent. However, each plantlet needed to be treated individually, and only transferred to the next step when leaves had reached the appropriate size. Transfer before this generally resulted in total plantlet death through desiccation, or tissue damage which retarded the ex vitro acclimatization process. Once the most recent leaf flush had produced leaves of approx. 15.0 cm in length at full expansion, plantlets were ready to be moved to RH conditions of 10.0 – 20.0 % (in the greenhouse). At this side, plantlets were beginning to resemble seedlings in size and phenotype (leaf size in 4 mo old seedlings was approx. 15.0 – 22.0 cm), and could also be exposed to increased light (300 µmol $m^{-2}s^{-1}$) without tissue damage.

**Acclimatization to increased light**

In preliminary work it was seen that plantlets would only respond to increased light levels after acclimatization to RH. It may be possible to move plantlets to increased light earlier than the 15.0 cm leaf size utilized in this protocol. However, this was not investigated. Plantlets moved from greenhouse conditions were first grown under shade cloth at 300 µmol $m^{-2}s^{-1}$ for 2 wk, or until the emergence and full expansion of the newest leaf flush. Then plantlets were transferred to 600 µmol $m^{-2}s^{-1}$ and maintained under these conditions until field transfer. Direct transfer of plantlets from the growth chamber (100 µmol $m^{-2}s^{-1}$) conditions to 600 µmol $m^{-2}s^{-1}$ without this intervening period resulted in leaf damage. Once moved to 600 µmol $m^{-2}s^{-1}$, tissue culture derived transplants are phenotypically nearly indistinguishable from the greenhouse-grown seedlings.

Acclimatization rates were very high (> 90.0 %) using this two-step protocol. Similarly high acclimatization rates (50.0 – 90.0 %) have been reported in Stage IV of *C. sativa* axillary

shoot culture (Vieitez et al. 2007). Acclimatization rates are generally noted as genotype-specific in *C. sativa*, which was not observed in this study. However, prevailing protocols for Stage IV of *C. sativa* acclimatize rooted clones in seedling trays containing many individuals. As a result, individualized plantlet care independent of genotype, as recommended in this study, could not be conducted with *C. sativa*. It is likely that with such groupings, only genotypes with highly synchronized development across clones would have high successful acclimatization rates. Although individualized care is labor intensive, this level of input can be justified in the conservation of rare and engendered species such as *C. dentata*.

Once acclimatized to higher light, transplants could be maintained in the greenhouse for several years (3 yr in this study). Formation of male catkins was even observed on some trees (Figure A.5). Plagiotrophic growth occurred in some specimens (Figure A.6) and could be controlled through pruning of horizontally growing shoots, and staking new growth to train it vertically. Once this new growth became lignified, apically dominant growth could continue without further staking. Plants treated this way maintained apical dominance after 2 yr of growth in the field (the length of time observed in this study).

Transfer to the field was best accomplished in the late fall (September – October). Leaves formed in the greenhouse (600 µmol $m^{-2}s^{-1}$) could not acclimatize to light levels in the field (> 1000 µmol $m^{-2}s^{-1}$). When plants (transplants) were moved into the field in the spring, leaves died or exhibited leaf burn. Most did not produce new leaves during that growing season. Transplants moved during the fall also lost leaves. However, fall transplants then entered winter dormancy. Both spring and fall transplants produced new leaf flushes the following spring. Both groups have continued to grow over 3 yr (the period of time observed in this study) and losses have not been observed to date in any of the field transferred plants followed during this period (n = 54). Many transplants (approx. 99) were donated by this project to other growers, and were no longer under direct observation.

***Fertilization***

During acclimatization of *C. dentata*, plantlets reach certain growth stage milestones defined by leaf length in the newest leaf flush. In this regard, fertilizer frequency had a significant effect on plantlet survival during acclimatization. Once rooted, plantlets required frequent and regular fertilization. Without this input, vegetative growth in plantlets stalls after rooting, and plantlets failed to acclimatize. However, as long as rooted plantlets were alive, their growth could

be stimulated through fertilization. Even after 2 mo without fertilizer, and almost no growth occurring, addition of fertilizer was generally found to induce rapid growth in plantlets. It was common to see micronutrient deficiencies (in particular Ca and Mg) develop between 4 – 6 wk after rooting, unless these micronutrients were applied.

Fertilizer used in this work included 30-10-10 (Plant-Products, Quebec, Canada), a 15.5-0-0 + 19 Ca (calcium nitrate product, Plant-Products, Quebec, Canada), and a micronutrient solution developed for hydroponic vegetable production systems (Plant-Products, Quebec, Canada) containing 4.7 % Mg, 7.0 % chelated iron (Fe), 0.4 % chelated zinc (Zn), 0.1 % chelated copper (Cu), 1.3 % B, 0.06 % molybdenum (Mo), and 40.0 % ethylene diamine tetra-acetate (EDTA). After rooting, plantlets were fertilized every 2 wk with full strength 30-10-10, and every 3 wk with 3.5 g/L calcium nitrate (not in the same week as the 30-10-10), and every 5 wk with a foliar feed of 0.3 g/L of micronutrient solution. This rate and frequency was maintained from immediately after initial rooting, to transfer into the field.

**8.1.3 Outline of the final protocol, optimized at the level of the individual**

The full protocol recommended for the acclimatization of *C. dentata* is summarized in Figure A.7. In brief, 6 – 7 wk old microshoots ≥ 3.5 cm in height are cut from the vascularized callus formed in vitro. Cut microshoots are dipped into Stim-Root #3 (0.8 % IBA), and planted under a covered tray filled with G6 potting mixture and fertilized with half strength 30-10-10. Microshoots are left in this container for 3 wk to form roots. After 3 wk, rooted plantlets are transferred to separate covered pots in G6 for individualized treatment, and fertilized with full strength 30-10-10. After 2 wk, plantlets are fertilized again with 30-10-10. After 1 more wk, plantlets are fertilized with calcium nitrate. After the production of leaves ≥ 10.0 cm in height (between 4 – 6 wk), covers are removed from the pots. At 5 wk, plantlets are given a foliar feed of micronutrients. Once the newest leaf flush is of ≥ 15.0 cm in length (between 1 – 2 wk), plantlets can be transferred to shaded conditions (300 µmol $m^{-2}s^{-1}$) in the greenhouse. It is important to ensure that the fertilizer regime is maintained during this period. After the production of 1 more leaf flush (between 1 – 2 wk), plants are ready to be moved to full light (600 µmol $m^{-2}s^{-1}$) greenhouse conditions. Plants can be maintained under these conditions, with a continued fertilizer regime, until field transfer.

### 8.1.4 Conclusion

*C. dentata* remains a challenging species to acclimatize ex vitro from axillary shoot culture. The protocol described here requires 10 – 14 wk for acclimatization from Stage II to greenhouse conditions, a very lengthy Stage IV. As well, care must be directed at the level of the individual, which is rare in commercial horticulture. However, the protocol described here has a relatively high success rate with the genotypes tested. Rooting ex vitro remains genotype-dependent, and it may be that different IBA concentrations or abiotic treatments would increase rooting rates in some of the more recalcitrant genotypes (Oakes et al. 2013). Success in the acclimatization of rooted plantlets was not dependent on genotype, and was highly successful overall (> 90.0 %). To date, > 150 clones of *C. dentata* from genotypes AcGraft, ASH1, Ellis 1, LCEM, New 11, New 16, and New 17 have been transferred into the field utilizing this protocol. It is hoped that the detail provided here, as well as the inclusion of observations on which factors were not successful, will aid in the ex vitro acclimatization of *C. dentata* in other research programs and contribute to the conservation of this species in the wild.

**Table A.1**. Relative rooting rates, and those not assessed, for all genotypes examined in Chapter 3. Genotypes AcGraft, ASH1, Ellis1, LCEM, New 11, New 16, and New 17 were successfully transferred to field conditions.

| Responsive | Variable | Recalcitrant | Non-Responsive | Not Assessed |
|---|---|---|---|---|
| AcGraft | ASH1 | Dundas | Hamilton Drive | Alessi |
| New 11 | | Ellis1 | New 15 | Deacon #2 |
| New 16 | | LCEM | | Deacon #5 |
| | | Light | | IIPP |
| | | Cemetery | | Metter |
| | | New 17 | | New 19 |
| | | | | Siebenmorgen |
| | | | | Ward |
| | | | | 664 |
| | | | | Zoar |
| | | | | Zrite |

**Figure A.1.** Stages in the axillary shoot culture of *C. dentata* (as described in Chapter 3).

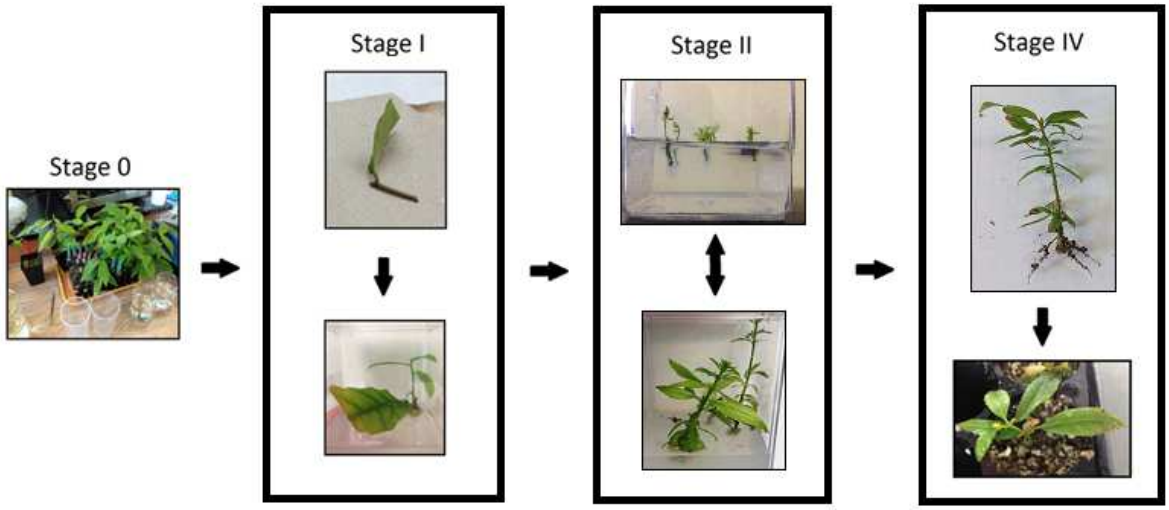

**Figure A.2.** Schematic of the treatment steps investigated in the development of a Stage IV protocol for axillary shoots of *C. dentata*.

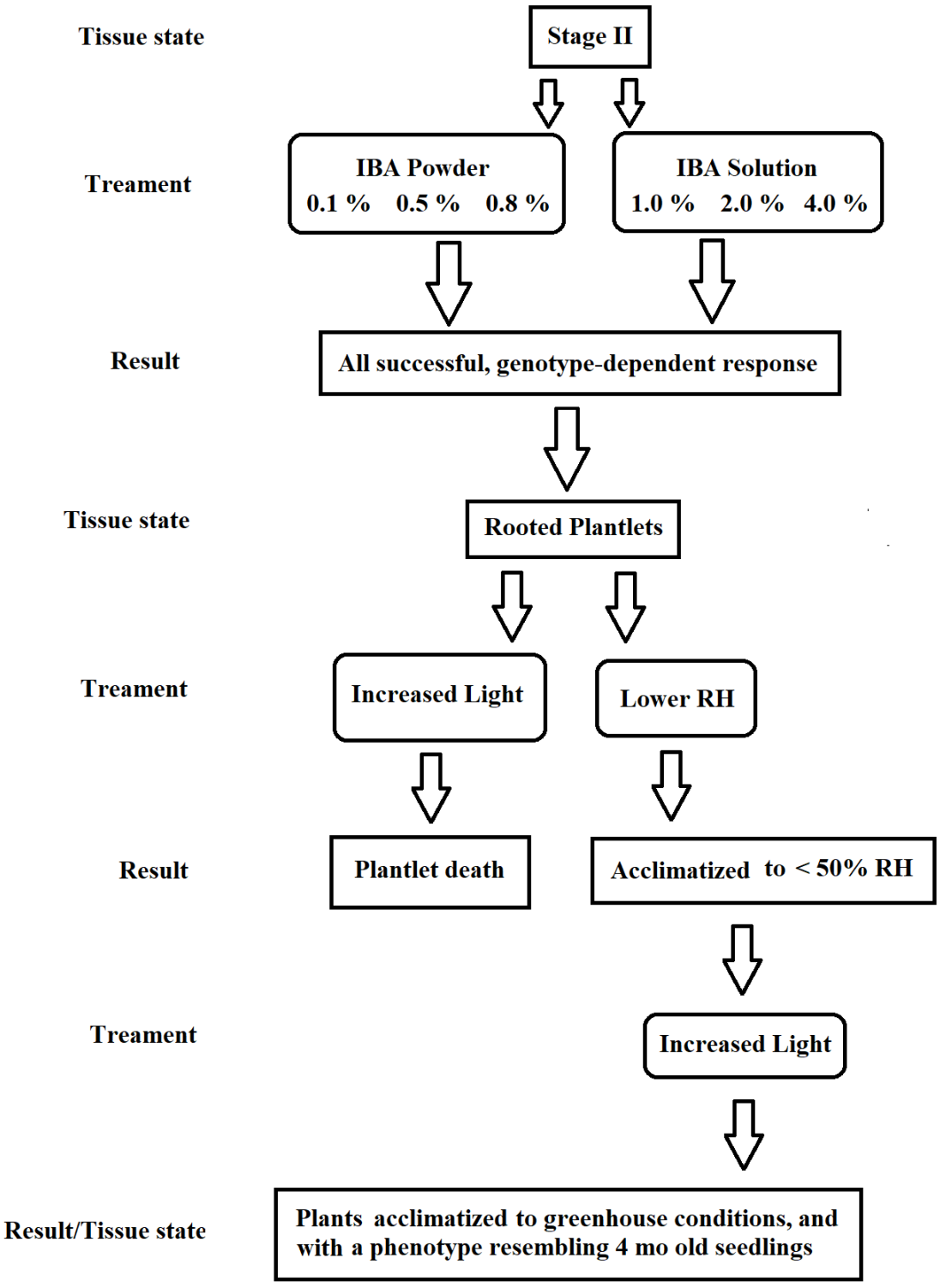

**Figure A.3.** Leaf damage (large arrows) resulting from increased light. Undamaged leaf tissue can also be seen (small arrow).

**Figure A.4.** Rooted plantlet of AcGraft, with successive leaf flushes increasing in size.

**Figure A.5.** Male catkin production in a tissue culture derived clone of ASH1, 1.5 yr after rooting ex vitro.

**Figure A.6.** Plantlets of ASH1 with plagiotrophic (left) and apically dominant (right) growth, 18 wk after rooting ex vitro.

**Figure A.7**. Summary of the full rooting and acclimatization protocol developed in this work.