In vitro mansonone production and vascular staining in populations, trees within populations and individual progeny of *Ulmus americana* L. in relation to Dutch elm disease

by

Fred G. Meier

A Thesis Submitted to

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In Partial Fulfillment of the

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

TABLE OF CONTENTS ii

LIST OF TABLES v

LIST OF FIGURES vii

LIST OF APPENDICES ix

ABSTRACT x

INTRODUCTION 1

LITERATURE REVIEW 3

Biology of *Ulmus americana* 3

History of Dutch Elm Disease 5
- Europe 5
- North America 8
- Canada 8
- Origin 9

Annual Disease Cycle 10

Management of Dutch Elm Disease 14

Cerato-Ulmin: Fungal Toxin 16

Host Resistance 17
- Anatomical Factors 17
- Sealing Vessels After Infection 18
  - Vascular Blockage 18
  - Compartmentalisation 20
- Phytoalexins 21
  - History and Introduction 21
  - Elicitors 22
  - Phytoalexin Toxicity 23
  - Phytoalexin Measurement 24
  - Use of *in vitro* Cultures to Measure Phytoalexins 25
- Mansonones 26

Mansonones and Elm Resistance.......... 27
Toxicity of Mansonones.......... 31
Elicitation of Mansonones.......... 32
Tissue Culture and Mansonones.......... 33
Extraction and Analysis of Mansonones.......... 34

CHAPTER 1.......... 36
INTRODUCTION.......... 37
MATERIALS AND METHODS.......... 38
Chemicals.......... 38
HPLC Quantification of Mansonone Standards.......... 38
Development of HPLC Technique.......... 38
Mansonone Standard Curve Development.......... 39
Callus culture.......... 40
Inoculation of callus.......... 40
Extraction of mansonones.......... 40
RESULTS.......... 42
HPLC System.......... 42
Measurement of Samples.......... 43
DISCUSSION.......... 47

CHAPTER 2.......... 49
INTRODUCTION.......... 50
MATERIALS AND METHODS.......... 52
Population Selection (collection sites).......... 52
Parent Tree Selection.......... 53
Seed (fruit) Collection.......... 53
Seed Germination and Seedling Growth.......... 53
Mansonone Production Determination.......... 54
Replicated analysis of mansonone production by genotype.......... 54
Mansonone production within populations and trees within populations.......... 55
Callus Tissue Production and Inoculation With *O. novo-ulmi*.......... 55
Inoculation of Seedlings With *O. novo-ulmi*.......... 55
Establishment of seedlings at Carman site.......... 56
Establishment of seedlings at Bird's Hill site.......... 56
Ophiostoma novo-ulmi culture and inoculation technique.......... 57
Seedling symptom measurement.......... 57
Statistical Analysis.......... 58
RESULTS.......... 60
Genotype Variation in Mansonone Production.......... 60
Mansonone Production Among Population and Parent Trees Within Populations.......... 65
Inoculation of Seedlings With *O. novo-ulmi*.......... 72

Relationship Between Vascular Staining and Mansonone Production...... 73
DISCUSSION.. 82

CHAPTER 3.. 89
INTRODUCTION...90
EXPERIMENT 1..90
MATERIALS AND METHODS.. 90
Callus Culture...90
Inoculation of Callus.. 91
Extraction and Analysis of Mansonones... 91
RESULTS..91
EXPERIMENT 2..93
MATERIALS AND METHODS.. 93
RESULTS..93
DISCUSSION.. 94

SUMMARY AND CONCLUSIONS... 98

REFERENCES.. 103

APPENDICES.. 114

LIST OF TABLES

Table		Page
1.1	Effects of varying solvent concentration on mansonone elution characteristics	42
1.2	UV spectrum for mansonones E, F, and G	43
1.3	Mansonone concentrations (E, F, and G) for sample calli from progeny of various trees	47
2.1	Unadjusted mean mansonone production (mansonones E, F, and G combined) for progeny from individual trees within populations	67
2.2	Mean unadjusted levels (µg/g) of individual mansonones (E, F, and G) for progeny from individual trees	71
2.3	Unadjusted mean mansonone levels (mansonones E, F, and G combined) for different American elm selections and elm species	72
2.4	Unadjusted mean levels of mansonones E, F, and G for progeny from American elm selections and elm species. N = number of progeny tested	72
2.5	Mean percent vascular staining for progeny from trees within population Beausejour (Beau). N = number of progeny per parent tree	75
2.6	Mean percent vascular staining for progeny from trees within population Henderson Highway (Hend). N = number of progeny per parent tree	76
2.7	Mean percent vascular staining for progeny from trees within population Lorette (Lort). N = number of progeny per parent tree	77
2.8	Mean percent vascular staining for progeny from trees within population Selkirk (Selk). N = number of progeny per parent tree	78
2.9	Mean percent vascular staining for progeny from trees within population St. Anne (Stan). N = number of progeny per parent tree.	79

Table Page

2.10 Mean percent vascular staining for progeny from trees within population Winnipeg (Wpg). N = number of progeny per parent tree.......... 80

2.11 Mean percent vascular staining for progeny from trees of American elm selections and various elm species. N = number of progeny per parent tree.......... 81

3.1 Mean production (± standard error where applicable) of mansonone (total E, F, and G) in *U. americana* callus cultures treated with various medium components including a water control and a treatment with no added components.......... 92

3.2 Mean production (± standard error where applicable) of mansonone (total of E, F, and G) in *U. americana* callus cultures with no added elicitors.......... 94

LIST OF FIGURES

Figure		Page
1.0	Chemical structures of mansonones found in *U. americana*.............	28
1.1	Standard curve for mansonone E derived through HPLC analysis. Y = -2.38 + 0.60X. Where Y = mansonone peak height(mm) and X = mansonone concentration (μg/mL). r^2 = 0.99............................	44
1.2	Standard curve for mansonone F derived through HPLC analysis. Y = -0.21 + 1.30X. Where Y = mansonone peak height (mm) and X = mansonone concentration (μg/mL). r^2 = 0.99............................	45
1.3	Standard curve for mansonone G derived through HPLC analysis. Y = -4.06 + 2.00X. Where Y = mansonone peak height (mm) and X = mansonone concentration (μg/mL). r^2 = 0.99............................	46
2.1	Flow diagram showing expected steps in analysing elm material......	59
2.2	Mansonone levels of progeny of 12 parent trees from population Beausejour. Two pieces of callus tested per progeny.......................	61
2.3	Mansonone levels in progeny from a single tree in the Beausejour population (Beau-1)..	62
2.4	Mansonone levels in progeny from a single tree in the Henderson Highway population (Hend-9)..	63
2.5	Mansonone levels in progeny from a single tree in the Winnipeg population (Wpg-15)...	64
2.6	Unadjusted mean mansonone levels (mansonones E, F, and G combined) for six populations of *Ulmus americana* within Manitoba. Means were not significantly different based on ANCOVA with callus weight as the covariate, P = 0.11..................	66
2.7	Relationship between mansonone production and callus weight for all samples analysed for mansonone production................................	68
2.8	Relationship between log mansonone production and callus weight for all samples analysed for mansonone production. Y = -4.06 + 2.00X. r^2 = 0.33...	69

Figure		Page
2.9	Relationship between vascular staining and mansonone production. Data points represent progeny tested from one parent tree. However, staining data was obtained from different progeny than used for mansonone production.	74

LIST OF APPENDICES

Appendix		Page
Appendix A:	B_5 plant medium used for callus growth of *Ulmus americana*..	115
Appendix B:	Takai and Richard's medium used for *Ophiostoma ulmi* culture...	116
Appendix C:	Analysis of variance (ANOVA) tables....................................	117
Appendix D:	The effect of cotyledon removal from seedlings of *Ulmus americana* on total height of seedling....................................	121
Appendix E:	The effect of callus size on the mansonone production ability of large pieces of calli..	124

ABSTRACT

Meier, Fred G. M.Sc., Department of Plant Science, University of Manitoba.
In vitro mansonone production and vascular staining in populations, trees within populations and individual progeny of *Ulmus americana* L. in relation to Dutch elm disease. Major Professor: Dr. W.R. Remphrey.

Dutch elm disease (DED) is a vascular wilt disease which has decimated populations of American elm (*Ulmus americana* L.) throughout North America. Mansonones are a family of phytoalexins found within the genus *Ulmus* and are thought to be involved in the resistance mechanism to DED. There have been no previous studies which have investigated the variation in production of mansonones within a large group of related elm. The purpose of this study was to investigate the production of mansonones within progeny from several parent trees within 6 populations of American elm located in Manitoba, Canada.

In this study a method to measure mansonones by high performance liquid chromatography was developed with a shorter run time and a simple solvent system procedure than previously published by other researchers. Mansonone production varied significantly between genotypes. Production of mansonones within progeny from one parent within a population were significantly different than other parents within the same population. No significant differences in mansonone production were found between populations.

Throughout the study when seedlings were measured for mansonone production through *in vitro* callus cultures it was found that mansonones were also produced in control treatments. Further experiments performed to address this problem revealed that mansonone production was initiated approximately at the same time callus tissue was

formed. No previous studies have reported this incidence.

Vascular staining, a symptom of DED, within progeny varied significantly between parents and populations. When mansonone production and vascular staining were compared among progeny from the same parent trees and populations, no correlation was found ($r^2 = 0.01$). At the juvenile stage, it appears that neither mansonone production nor vascular staining is an adequate indicator of resistance.

This is the first study to show large differences in the production of mansonones over such a large study group. Future research on mansonone production and DED resistance must concentrate on more mature material which can show visual symptoms of DED more accurately.

INTRODUCTION

The American elm (*Ulmus americana* L.) is a native tree to much of North America. The American elm has also been used for urban plantings, and is well adapted to the climate of the northern prairies. The American elm possesses good form and resistance to many urban stresses. A major liability to the American elm is that it is very susceptible to Dutch elm disease (DED), a fungal vascular wilt disease which is lethal. The disease is capable of killing large numbers of American elm within a year. Provincial and civic governments management efforts have slowed the annual loss of American elm in Manitoba to approximately 2% annually in areas were DED is actively managed (Westwood 1991). However, despite these efforts the long term future of the American elm may lie in the development of genotypes with increased resistance to the disease.

Host resistance within the elm species has been explored for many years. Through conventional selection techniques some resistant American elms have been produced (Smalley *et al.* 1993). The limitation with conventional techniques of breeding and selection for resistance is that it takes many years to select an elm resistant to the disease. The primary reason for this long selection time is that trees must be at least 3-4 years old before they show symptoms after infection. Therefore, a selection program based on indicators of resistance, which could be tested at a young age, would be less time consuming in the search for resistance and therefore shorten the breeding cycle.

Mansonones are a family of fungitoxic compounds produced by the genus *Ulmus*

after wounding or infection. These compounds are also produced after an elm is infected by DED (Proctor and Smalley 1988) Concentrations of these mansonones are toxic to the DED fungus (*Ophiostoma ulmi*) *in vitro* (Wu *et al.* 1989). One theory of host resistance within elm suggests that if an elm can be selected for a rapid increase in production of mansonones quickly after infection by DED, trees could be resistant to the disease (Duchesne 1993).

There have been no previous reports on the genotypic variation among a large population of elm with respect to their mansonone production abilities. Such information would be beneficial in further developing the idea of breeding for resistance to DED based on the production of mansonones. The objectives of this research were to develop a technique where mansonones could be measured quickly and accurately, therefore allowing for a large amount of samples to be analysed. The second objective was to measure the production of mansonones from callus tissue derived from a large number of genotypes selected from several populations within Manitoba. The third objective was to relate the mansonone production numbers to indicators of resistance such as vascular staining within seedlings of similar parentage.

LITERATURE REVIEW

Biology of *Ulmus americana*

The genus *Ulmus* consists of deciduous tree species within the north temperate regions of the world. In North America the species range from Mexico to Newfoundland and west to Saskatchewan. They do not occur naturally west of the Rockies in Canada (Fowells 1965; Britton and Brown 1913; Hosie 1979). The American elm, *Ulmus americana* L., is found throughout the range of *Ulmus* in North America.

Ulmus americana has been variously called the American, grey, soft, swamp, water or white elm (Hosie 1979; Dirr 1983). It is a large deciduous tree growing to 40 m in height and 3.3 m in basal main-stem diameter, with a life span of 175-200 years, reaching maturity at 150 years. Nevertheless, specimens of 300 years have been reported (Hosie 1979; Fowells 1965). The tree has gradual spreading branches forming a wide expanding crown giving the tree its typical vase or umbrella shape, though crown shapes do vary between genotypes (Hosie 1979; Dirr 1983). *U. americana* has grey scaly, shredding bark which is deeply fissured and furrowed into intersecting channels (Dirr 1983).

The foliar and reproductive characteristics of *U. americana* are consistent with those of the genus. Leaves are oval or obovate with an acuminate tip, alternate, simple, dark glossy green, pinnately veined, smooth or rough above, pubescent or becoming glabrous underneath, 5-13 cm long and 4-8 cm wide; petioles are 5-8 mm long. Buds are ovoid, obtusish or acute and there is no true terminal bud because it aborts naturally and

the next lateral bud replaces it. Flowers are produced before leaves emerge and are borne on a 1-2 cm long filiform pedicel. Flowers are perfect and greenish red in colour, in fascicles of 3-4. Stigmas are white and have a drooping form (Britton and Brown 1913; Hosie 1979; Bailey and Bailey 1976).

Ulmus americana is found growing in a variety of climates, from humid and warm conditions in the southwest to arid and cool conditions in the northwest. Average maximum summer temperatures range from 30 to 35°C and average minimum temperatures range from -40 to 0°C. Rainfall is generally not limiting to growth, except for occasional drought conditions in the prairie regions. Precipitation ranges from an annual low of 35 cm in North Dakota and Manitoba to a high of 75-87 cm in the southeastern areas. Frost free days range from a low of 80-100 in the north to 280-320 in the south (Fowells 1965).

Ulmus americana prefers rich, moist, well drained sandy loam or gravelly soils, but also quite commonly is found on flood plain areas where it tolerates silty soils and spring flooding. In its native habit it is a river bottom forest tree and it thrives on stream banks and river forests in a mixture with other trees or in pure stands. Single specimen trees are also quite common in the wild. *U. americana* tolerates a pH range from 5.5 to 8.0.

Ulmus americana can tolerate various stresses including variable pH, drought, and salt. Such resistance coupled with a quick growth rate and appealing form has led to the frequent use of this tree for urban plantings. Throughout the northeastern U.S.A, southern Ontario, Quebec and Manitoba the American elm at one time was the exclusive tree

planted in urban settings. It was highly regarded as one of the best boulevard trees available. Cities such as New York, Toronto and Winnipeg planted large numbers of American elm on street settings, and when planted on opposite sides of a street the elms would create a gothic arch effect. However, *U. americana* is presently not recommended for planting in urban settings because of its susceptibility to Dutch elm disease (DED). This monoculture of elms in many cities was decimated by the movement of the DED from Europe to North America in the 1930s (Stipes and Campana 1981; Fowells 1965; Bailey and Bailey 1976; Britton and Brown 1913; Dirr 1983; Hosie 1979).

History of Dutch Elm Disease

Europe

DED is a vascular wilt disease of elm caused by a fungal pathogen (*Ophiostoma ulmi*) which is transmitted by its vector the elm bark beetles. DED has the ability to rapidly kill an elm. DED was first noticed in northwestern Europe where there were many reports of massive deaths of the native European field elm (*Ulmus carpinfolia*). The origin of DED in Europe is not known because when the disease first appeared, World War I was in progress. During the war people were not greatly concerned with the elms thus few records are available from this time. Also, the strain of the DED pathogen was very weak and would only affect a few very susceptible elms. These two factors could have caused DED to go unnoticed for a period of time. The Netherlands is generally considered to be the first European country in which DED was observed, but

reports of simultaneous outbreaks in France, Holland, Belgium, and Germany all within three years of each other indicate that the focal point of origin is not clear (Gibbs 1978).

The identification of the causal agent and general description of the disease was primarily undertaken in the Netherlands. In 1919, Schwarz (a Dutch pathologist) isolated a fungus from a diseased elm which resembled a synnema (coremium) in its sexual form . In 1921 Spierenburg published the first report of DED and reported it as an unknown disease of the elms. The following year Schwarz concluded DED was the result of a fungal invasion, and named the fungus *Graphium ulmi* (Holmes 1990; Holmes and Heybroek 1990; Stipes and Campana 1981). The perfect stage initially described as *Ceratostomella ulmi* Buism. in 1932 by Buisman was changed to *Ophiostoma ulmi* in 1934 by Nannfeldt and then to *Ceratocystis* in 1952 by Moreau (Stipes and Campana 1981; Brasier 1991; Holmes 1990; Holmes and Heybroek 1990). The name *Ceratocystis ulmi* became quite popular and was the primary designation used in many scientific publications for approximately the next 30 years. In 1984 de Hoog and Scheffer distinguished the *Ophiostoma* and *Ceratocystis* fungi into two groups. Since then *Ophiostoma ulmi* (Buism.) Nannf. has been the recognized nomenclature for the DED pathogen (De Hoog and Scheffer 1984).

The disease spread from northwestern Europe to the rest of Europe in the following years and by the 1960s the disease ranged from the Ural mountains in the Soviet Union to Ireland but occurred only in small amounts in the Scandinavian countries. The disease also occurred in Asia but only in three locations (Gibbs 1978). In the early 1930s DED then spread to North America (see next section).

In 1971 in Britain it became apparent that some regions previously stricken with DED were now once again being attacked, but this time with greater losses than occurred in the initial epidemic (Gibbs and Brasier 1973; Gibbs 1978). The idea of two separate strains of *O. ulmi* was emerging and it appeared that the second epidemic was the result of an aggressive strain (Gibbs *et al.* 1972). Differences in the two strains is evident when cultured *in vitro*. The aggressive strain was shown to be fast growing with aerial mycelia aggregated into radial strands showing distinct zones when exposed to light. The non-aggressive strain was slow growing, had a waxy yeast like appearance, sparse mycelium and showed little zonation when exposed to light (Gibbs and Brasier 1973). Thus, these two strains were separated into the aggressive and non-aggressive subgroups of the *O. ulmi* pathogen (Brasier 1982). The aggressive subgroup was further separated into the North American race (NAN) and the Eurasian race (EAN). Brasier divided the aggressive and non-aggressive subgroups of *O. ulmi* into two separate species, the non-aggressive species was named *O. ulmi* (buism.) Nannf. syn. *Ceratocystis ulmi* (Buism.) Moreau and the old aggressive subgroup was designated *O. novo-ulmi* Brasier sp. nov. (Brasier 1991).

The most aggressive strain of the fungus in Europe actually came from North America. It is presumed that in the mid 1960s a shipment of logs of rock elm (*Ulmus thomassi*) from Canada transported the aggressive strain of the fungus to Britain (Brasier and Gibbs 1973; Gibbs 1978). Throughout the 1970s this aggressive strain caused a second epidemic of DED much more severe than the first. Subsequent large losses of *U. carpinfolia* and *U. laevis* in Europe and also *U. pumila* var. *arborea* in central Asia is evidence of the severity of the aggressive strain (Gibbs 1978; Stipes and Campana 1981).

North America

DED became established in North America in the late 1920s and early 1930s. The initial and perhaps most serious infection occurred in New York City. It is suspected that a shipment of elm logs from Europe carried the pathogen overseas. The presence of an established breeding population of *Scolytus multistriatus* (Marsham) (European elm bark beetle) as well as a large population of *Hylurgopinus rufipes* (Eichhoff) (Native elm bark beetle) and the presence of a very susceptible host *U. americana* provided the pathogen with a situation in which to thrive (Gibbs 1978; Stipes and Campana 1981).

By the 1950s areas of initial infection increased and coalesced forming a westward moving DED front. In the U.S., during World War II, the efforts of DED control were dropped and resources focused on the war. The disease then became more entrenched in the U.S. and spread westward. By 1968 the disease had spread to the eastern side of the U.S. well out of the natural range of the American elm (Stipes and Campana 1981), within Canada the disease had spread from the maritimes to Manitoba.

Canada

In Canada the first outbreak of DED was reported in Sorel, Quebec in 1942. This outbreak was probably from an independent infection introduced from Europe some time earlier. From the initial infection in Sorel the disease spread throughout the province of Quebec, whereas other areas of infection within Canada such as the Maritimes, Ontario and Manitoba originated from inoculum carried directly from the U.S. (Stipes and Campana 1981; Campana and Stipes 1981). In 1975 DED was detected at three sites in

Manitoba; Winnipeg, Brandon and Selkirk. Surprisingly these areas were not close to the U.S. border and were geographically isolated from each other. The disease probably was introduced by firewood brought in by campers from an infected area (Westwood 1991). The year prior to the initial outbreak of the disease there was a camper convention in Manitoba and this was thought to be the source of the initial inoculum.

The disease moved from the initial areas of introduction throughout Manitoba and now occurs throughout the natural range of the American elm in this province. The disease has progressed westward into Saskatchewan and in 1992 there were 14 sites of infection within the province (Cerezke and Brandt 1993). Although DED has not yet reached Alberta, the elm bark beetle was found in Calgary for the first time in the summer of 1994 (Edmonton Journal 1994).

Origin

The evolutionary origin of DED has been a widely debated area. The most accepted theory suggests Asia as the centre of DED origin based on two pieces of evidence. Firstly, the high level of resistance among the Chinese elm species *U. pumila* (Siberian elm) and *U. parvifolia* (Chinese elm) suggests that the fungus co-evolved in this area in association with the hosts. The second piece of evidence for Asia as the centre of origin is the high level of species diversity in the *Ulmus* genus in this part of the world (Brasier 1990). The resistance of a species will be higher in areas where they evolved with the pathogen. The validity of the Asian hypothesis has been questioned because *U. pumila* is not resistant to DED throughout its entire range. Also, certain Asian elm

species are not resistant to DED while others are only slightly susceptible, and there have been no reports of DED ever occurring in Japan even though it contains two susceptible elms and is in close proximity to China (Brasier 1990).

There are several theories as to how the DED pathogen moved from Asia to Europe. The wicker basket theory involves the idea that Asian immigrant workers carried their possessions in elm wicker baskets during World War I, and introduced the disease to western Europe. Reports of the disease being prevalent prior to the movement of these immigrant workers into the immediate area has cast doubt on this hypothesis (Gibbs 1980).

Another theory dismisses Asia as the centre of origin. Instead the seemingly instantaneous arrival of DED to Europe was postulated to be a mutation caused by the mustard gases used in the war. This theory has followed a similar fate to the wicker basket theory. The use of mustard gases occurred well after the initial reports of the disease (Holmes 1990). Although many different hypotheses have been proposed to explain the origin of the disease it is still a mystery.

Annual Disease Cycle

Within Manitoba DED involves three components, the native American elm (*Ulmus americana*), the DED fungus (*Ophiostoma ulmi*), and the vector of the disease, the native elm bark beetle (*Hylurgopinus rufipes*).

Elm is at its most susceptible period in the spring (Banfield 1968). Active growth of the tree in the spring triggers the production of new vessels in the xylem. These

vessels are large in diameter to carry the additional amount of water needed for active spring growth. When *O. ulmi* enters the tree any spore can multiply through yeast-like budding, and this is the presumed way the fungus spreads through the xylem stream (Stipes and Campana 1981). The newly formed large diameter vessels allow uninhibited progression through the xylem (Takai *et al.* 1979; Elgersma 1970).

As the fungus spreads the elm reacts to a toxin produced by the fungus called cerato-ulmin. The elm attempts to slow or compartmentalise the fungus to one region to prevent further host colonisation by the pathogen. One mechanism is the production of tyloses and gels, to block vessels. If they are not produced quickly enough the fungus will proceed through the xylem, ahead of the tree's reaction. The xylem then becomes blocked with fungal spores, tyloses and gels which restrict the flow of water and cause wilting and eventually death (Elgersma 1970; Elgersma 1973; McNabb *et al.* 1970). The tree's reactions to infection can be beneficial if it reacts quickly enough and harmful if it does not.

Symptoms of DED are associated with different times of the year and stages of infection. After a healthy tree is infected with *O. ulmi*, the initial signs of infection are wilting in a portion of the crown. Later in the year leaves become chlorotic. The leaves then die and turn brown and often remain attached to the infected branch. When a portion of the crown displays these symptoms it is known as flagging. Flagged portions of the crown stand out against the healthy green portions of the crown and are easily detected. In the same or subsequent years the other portions of the crown will show symptoms and eventually the tree will die from a lack of water and nutrients because the

conducting tissues are plugged. The death of a tree can take anywhere from a few weeks to several years (Stipes and Campana 1981; Stack and Laut 1986).

Another symptom of an elm infected with DED is vascular staining. When the bark is removed from an infected branch, brown streaking is evident in the wood. This brown colour is a result of the occlusion of vessels and vessel groups with gums, gels and fungal extracts. Despite these visible symptoms, an accurate diagnosis of DED can only be accomplished through pathogen isolation from infected wood and the identification of *O. ulmi* as the pathogen.

The primary vectors of DED throughout the world are the elm bark beetles. Specifically within Canada there are two elm bark beetle species which are vectors of the disease: the native elm bark beetle, *Hylurgopinus rufipes* and the introduced European elm bark beetle, *Scolytus multistriatus*. The distribution of the native elm bark beetle bears a close relationship with the distribution of the American elm throughout North America. The smaller European elm bark beetle is most prominent in the eastern parts of Canada but seems to be moving westward (Ives and Wong 1988). In Manitoba the native elm bark beetle is the primary vector of DED, and can follow one of two separate lifecycles based on timing of its stages.

The majority of the beetles overwinter as adults in Manitoba (Ives and Wong 1988). Within the overwintering galleries constructed by the adult beetles in diseased trees, mycelia, conidia, synnemata and perithecia are present (Stipes and Campana 1981). The overwintering adult beetles emerge in the spring from their overwintering galleries with either conidia, coremial spores or ascospores adhering to them. The beetles then

move to the crown of the same elm or to adjacent elms and feed on the bark of large branches or the trunks (Gardiner 1981; Neill and Leatherman 1987; Ives and Wong 1988; Takai *et al.* 1979). At this point in the lifecycle infection can take place. Spring feeding coincides with the elm's most susceptible period to infection by *O. ulmi* (Gardiner 1981; Takai *et al.* 1979), that is when tree growth is at its peak.

After feeding in the spring the adult beetles are attracted to weakened trees where they proceed to lay their eggs. The eggs hatch in the same year. Adults then emerge, in late summer, feed briefly on healthy trees, and then create their overwintering galleries within healthy or newly infected trees. Some spread of the disease can occur from the new adult beetles emerging in late summer, but not as much as in spring.

H. rufipes may overwinter as larvae if the eggs are not laid in sufficient time to develop into adults. In this case adults fly to weakened elms which may be infected with DED later in the summer, burrow out brood galleries and lay their eggs. The eggs hatch in late summer and the larvae proceed to overwinter (Gardiner 1981; Takai *et al.* 1979). In the spring the larvae resume growth and adults eventually emerge. If the brood tree was infected the adults carry spores of *O. ulmi*. They proceed to feed on healthy elms spreading the disease. The majority of the DED spread is a result of the overwintering adults as opposed to the overwintering larvae, because overwintering as adults is more common (Gardiner 1981; Takai *et al.* 1979; Landwehr *et al.* 1981; Ives and Wong 1988). There has been little evidence of overwintering larva in Manitoba (Westwood, person. comm. 1995).

The attraction of the beetles to weakened or injured elms is related to the secretion

of volatile substances by the trees. These trees thus become the brood trees and are the source points for next years DED infection (Byers *et al.* 1980; Landwehr *et al.* 1981). Elm volatiles are also secreted whenever an elm is pruned within the growing season. Several studies have shown an increased visitation to pruned elm branches by elm bark beetles (Byers *et al.* 1980; Landwehr *et al.* 1981). Therefore it is recommended that elm pruning be done in the fall or in the winter when beetle activity is much lower or arrested.

Management of Dutch Elm Disease

There is no cure for DED. Management is a more appropriate term than is control, because the disease itself cannot actually be controlled. Instead, we can manage its severity. The management practices which account for the relatively low incidence rate of disease in Manitoba (approx. 2% yearly) must be continued on an annual basis or the rate of disease spread will increase again (Cerezke and Brandt 1993; Westwood 1991).

Sanitation is the most efficient technique to manage DED on a large scale. An important component of the management scheme is a yearly survey program which identifies and marks infected trees for removal. Sanitation involves the timely removal of all infected trees and elm wood which would perhaps serve as elm bark beetle breeding sites. Removal should be implemented in the winter when trees are dormant and beetles contained within the diseased trees. The Manitoba provincial and civic governments have imposed laws preventing citizens from storing elm firewood with the bark left on and of pruning elms during the beetles' active period in the spring or summer which would

attract them to the wounds (Byers *et al.* 1980; Landwehr *et al.* 1981). Elm wood can only be utilized for firewood if the bark is removed, thereby rendering it uninhabitable to the beetles.

The sanitation program also involves the regular removal of dead wood and general pruning of boulevard trees at the appropriate time of year. Removal of dead branches reduces potential brood sites. The general pruning allows the tree to remain in good health and therefore reduce infection. Removed elm wood is either buried, burnt, or if large enough milled into lumber (Westwood 1991).

Reduction in the elm bark beetle population within communities is accomplished by the application of an insecticide to the bottom 1-2 metres of healthy trees, prior to beetles moving to the base of trees to overwinter. The insecticide is applied directly to the trunk. The insecticide used in Manitoba is a 0.5% chlorpyrifos solution which is generally applied in August and September to prevent beetles from overwintering.

Another management technique is the injection of fungicides into the vascular system of elms to prevent fungal infection and growth. This technique is quite costly and only lasts between 1-3 years. The cost involved in this injection limits it to private or important specimen trees. Registered fungicides commonly used to control DED are Lignosan™ or Arbotec 20™ (Murray and Dickson 1994).

Diseased trees are replaced with either different species of elm or different trees. The approach is not to plant susceptible elms in large numbers. A monoculture of trees must be avoided so that another epidemic does not happen again.

Other elm species have shown high rates of resistance to DED. Some of these are

U. pumila and *U. davidiana japonica* (Japanese elm). The resistance in these two species is much higher than in American elm but the resistance in these species also varies with fungal strains. The drawbacks to using these species include less desirable growth characteristics and form, poor salt tolerance and susceptibility to winterkill and wind breakage (Westwood person. comm. 1995).

Cerato-Ulmin: Fungal Toxin

Cerato-ulmin is a phytotoxin produced by *O. ulmi*, and was first isolated in 1942 (science 95 512-513 1942). Cerato-ulmin produces symptoms similar to those of DED when inoculated into elm (Stevenson *et al.* 1979; Takai *et al.* 1983; Richards and Takai 1984). Cerato-ulmin is a small protein with a molecular weight of 13000 and is high in cystine, proline, levorine, serine, and aspartic acid, but is low in histidine, lysine, arginine, isoleucine, phenylalanine and tyrosine, and does not contain cysteine or tryptophan (Stevenson *et al.* 1979). This toxin functions at the cellular level and causes cells to change their rate of electrolyte loss, plasma membrane permeability and also respiration rate (Richards and Takai 1984; Richards 1993). A correlation between cerato-ulmin production and *O. ulmi* pathogenicity has also been found (Jeng *et al.* 1987). The higher the cerato-ulmin production within an isolate, the higher its pathogenicity (Jeng *et al.* 1987; Takai *et al.* 1983; Scheffer *et al.* 1987). Non-aggressive isolates produces very low levels of cerato-ulmin as compared to aggressive isolates, which produce large amounts (Brasier *et al.* 1990). The production of cerato-ulmin between EAN and NAN isolates is not different. Cerato-ulmin production is not the only factor involved in different levels

of aggressiveness (Brasier *et al.* 1990).

Host Resistance

Perhaps the best approach to controlling DED is through the use of host resistance. There are three general factors involved in elm resistance to *O. ulmi*: Anatomical factors which affect the extent to which the pathogen can spread through the elm, the ability of the elm to seal off the fungus to limit its spread through the tree, and the production of fungitoxic compounds (phytoalexins) to reduce fungal growth within the tree. These factors are additive in their function to increase host resistance (McNabb *et al.* 1970).

Anatomical Factors

Vessel characteristics such as a small diameter, shorter length, and lack of interconnections between vessels impede the travel of spores through the vascular system. Greater susceptibility in elms to *O. ulmi* has been correlated with increasing average xylem vessel size or size of groups (McNabb *et al.* 1970; Elgersma 1970). Elergsma (1970) found that vessels were shorter and more prevalent in a resistant clone as compared to a susceptible one. Vessel size and diameter are not static throughout the year and the seasonal changes in vessel dimensions also seem to correlate with susceptible and resistant periods within elms (McNabb *et al.* 1970; Elgersma 1970). The period of highest susceptibility was correlated with the dissolution of earlywood end walls (Banfield 1968). This dissolution of vessel end walls would allow for rapid transfer of conidial

spores throughout the vascular system. Similarly the onset of latewood production was correlated with seasonal resistance (Banfield 1968).

The higher degree of resistance in *U. pumila* is considered to be related to the fact that *U. pumila* initiates latewood production earlier than *U. americana* (Elgersma 1970). In addition, the amount of branching between different vessels is greater in *U. americana* than *U. pumila* allowing for tangential movement of spores between vessels (Elgersma 1970). McNabb *et al.* (1970) found a stronger correlation between disease resistance and vessel group size (product of average vessel diameter x average number of contiguous vessels) than individual vessel size and diameter.

Sealing Vessels After Infection

Vascular Blockage

Angiosperms are especially susceptible to vascular invasions because they need to move large amounts of water to sustain life. To survive exposure to such invasions, the plant must be able to stop or restrict microbial spread within its vessels. One mechanism to achieve this is by vascular blockage, that is by plugging vessels or groups of vessels by various means, thereby halting the spread of the fungus through the tree. Vascular blockage can either be accomplished by walling off certain vessels from others or by production of tyloses or gels to block the vessels (Shigo and Tippett 1981; Elgersma and Miller 1977; VanderMolen *et al.* 1977). Vascular blockage as well as

contributing to resistance to DED is also a reason why elms succumb to DED (see annual disease cycle). If the plugging of the vessels is not rapid enough to halt the fungal spread the blockage will only serve to impede water transport in the tree. If the tree's reactions are delayed the production of these vessel occlusions will always be too slow and ineffective. Obstruction of water transport leads to wilting and eventual death of the tree.

Tyloses are the enlargement and ballooning out of parenchyma cells through pit pairs of the vessels (Beckman 1971). These tyloses are formed as a result of mechanical or microbial injury, and function in plugging the vessels in an attempt to compartmentalize an area or vessel to halt the spread of a pathogen, as in the case of DED. Elergsma (1973) found a significantly higher number of tyloses being formed in a resistant elm clone as compared to a susceptible one (Elgersma 1973). Other evidence suggests that tyloses alone do not determine whether an elm is resistant or susceptible because non-aggressive and aggressive strains of the fungus were shown to have no difference in tylose production (Elgersma and Miller 1977). A study using a growth retardant to shorten vessel length also suggested that *O. ulmi* could have the ability to penetrate or circumvent tyloses either through enzyme degradation or lateral growth around a blocked vessel (Brener and Beckman 1968). Therefore, tyloses alone do not account for increased resistance in a host.

Vessel occlusions, compounds or physiological reactions which plug vessels, can be associated with other factors as well. For example, vascular gels have been shown to be associated with occlusion of vessel lumina (VanderMolen *et al.* 1977). These preformed gels swell when triggered by a fungal invasion. Plugging of elm vessels has

also been associated with fibrillar material and fungal cells (Ouellette 1978a; Ouellette 1978b; Ouellette and Rioux 1993).

Compartmentalisation

Compartmentalisation is a process by which the elm attempts to restrict the spread of the fungus within the tree, by limiting it to one area. Anatomical features, vascular blockage, and production of subsequent walls all act in the process of compartmentalisation. There are two components to compartmentalisation, walling off of injured and infected xylem as quickly as possible and walling off xylem at time of infection from new xylem produced (Shigo *et al.* 1980). Compartmentalisation halts the spread of the fungus throughout the vascular system.

The tissue generating and compartmentalisation capacity of the American elm varies greatly and is probably under genetic control (Shigo *et al.* 1980; Shigo and Tippett 1981). The process of compartmentalisation requires a substantial amount of energy to produce all the new tissues. If trees have low energy reserves because of new growth or seed production the tree may become more susceptible to DED (Domir *et al.* 1992). The susceptible period coincides with the time of beetle emergence from their overwintering galleries.

Phytoalexins

History and Introduction

The idea that plants produced defensive compounds as a result of infection was proposed by Muller and Borger (1941). The origin of phytoalexin is Greek meaning "to ward off in plants". Muller and Borger (1941) noticed that potato tubers initially inoculated with a non-pathogenic race of *Phytophthora infestans* developed localized resistance to a pathogenic race of the fungus. Thus the "phytoalexin theory" that plants accumulated chemicals inhibitory to development of infectious agents upon infection was developed (Kuc 1976).

Phytoalexins were not isolated until 1960, when Cruickshank and Perrin isolated pisatin, a phytoalexin from the pods of *Pisum sativum* infected with the brown rot fungus, *Monilinia fructicola* (Cruickshank and Perrin 1960). Since the first phytoalexins were isolated, they have been found in many plant families. Numerous phytoalexins are found within single families, but for the most part they are quite similar within families.

Since the first definition was formulated from the preliminary work, a more precise definition of phytoalexins has since been proposed, "Phytoalexins are low molecular weight antimicrobial compounds that are both synthesized by and accumulated in plants after their exposure to microorganisms" (Deverall 1982). This definition is also incomplete because phytoalexins can also accumulate under abiotic conditions such as UV radiation, temperature shock, wounding and treatments with organic salts (Harborne 1988; Kuc 1976).

Increased phytoalexin accumulation is related to elevated disease resistance in several different families of plants (Marley and Hillocks 1993; Latunde-Dada and Lucas 1985; Bowen and Heale 1987; Snyder *et al.* 1991; Liu *et al.* 1992; Hain *et al.* 1993; Yang *et al.* 1989; Hildenbrand and Ninnemann 1994). Phytoalexins accumulate quickly after infection in a plant which is resistant. A susceptible plant will be delayed in the production and total accumulation of phytoalexins after infection.

Elicitors

Phytoalexins can be elicited by many different agents including biotic and abiotic sources. Since phytoalexins are stress related defense metabolites, they are produced whenever the plant encounters a stress. In most cases phytoalexins are formed most frequently and in the highest amounts when plants are infected or parasitized by fungi (Bailey 1982).

An elicitor is a compound of the fungal cell wall which effectively induces the host plant to produce phytoalexins. The most efficient elicitors are fungal extracts. Several components, such as glycoproteins (Coleman *et al.* 1992; Renelt *et al.* 1993), glycopeptides (Basse *et al.* 1992; Basse *et al.* 1993), glucomannans (Basse and Boller 1992; Keen *et al.* 1983), chitin fragments (Ren and West 1992) and glucans (Ayers *et al.* 1976; Waldmuller *et al.* 1992) are efficient elicitors. Elicitors have also been isolated from the host plant, and are proposed as the elicitors of abiotic phytoalexin production (Bowen and Heale 1987).

The site of elicitor activity is generally thought to be the host plasmamembrane.

A glycoprotein elicitor isolated from *Phytophthora megasperma* f.sp. *glycinea* was shown to bind specifically to target sites on the parsley (host) plasmamembrane, thereby initiating a transmembrane signalling process which leads to transient activation of plant defense genes (Renelt *et al.* 1993). This activation of defense genes is the trigger of phytoalexin production.

Phytoalexin Toxicity

Phytoalexins are not only toxic to microorganisms, but have some toxicity across the biological spectrum. Phytoalexins will affect other plants and also animals. Cases of phytoalexins adversely affecting humans, mice, and nematodes have been reported. Phytotoxic affects of phytoalexins have also been reported including causing damage to potato and tomato cells (Smith 1982). However, most bioassays for phytoalexin toxicity are undertaken between host phytoalexins and common pathogens specific to that host. These results show toxicity of phytoalexins to pathogens (Proctor *et al.* 1994; Smith 1982). Toxicological studies undertaken with pathogens and phytoalexins have revealed that host phytoalexins are more toxic to pathogens which are not pathogenic to the host (Delserone *et al.* 1992; Proctor *et al.* 1994). The fact that phytoalexins from other plants are more toxic to unrelated pathogens has spawned the idea of producing transgenic plants with introduced phytoalexin capabilities. The ability to increase resistance with transgenic plants based on the addition of a foreign phytoalexin gene has been accomplished in tobacco (Hain *et al.* 1993).

The fungal membrane is one of the target sites for the toxicity of phytoalexins.

There are many examples of fungal membrane disfunction caused by host phytoalexins (Wu *et al.* 1989; Dmitriev *et al.* 1989; Smith 1982). Damage to the cell membrane is consistent with ion leakage. Other changes as a result of phytoalexins are: ribosomal aggregation, aggregation of electron dense material in the mitochondria (Wu *et al.* 1989), repression of DNA and RNA synthesis in bacterial cells (Dmitriev *et al.* 1989), inhibition of enzymes and plasmalemma damage (Smith 1982). The ability to affect membranes has lead to the understanding that phytoalexins must be of a lipophilic nature.

Phytoalexin Measurement

The extraction and measurement of the various types of phytoalexins varies greatly because the compounds differ so widely in their polarity, solubility, and their accessibility *in vivo*, making a single extraction or analysis technique impossible (Jeng and Hubbes 1980). The sample to be tested for phytoalexin content is usually extracted with an organic solvent and then the lipid extract is examined for the presence of phytoalexins. Chromatography is the most common technique for detection and isolation of phytoalexins. High Performance Liquid Chromatography (HPLC) (Snyder *et al.* 1991; Spring *et al.* 1992; Arnoldi and Merlini 1990; Gustine *et al.* 1990) and Thin Layer Chromatography (TLC) (Liu *et al.* 1992; Latunde-Dada and Lucas 1985; Ingham and Harborne 1976; Marley and Hillocks 1993; Lyon 1972; Moreau *et al.* 1992; Braga *et al.* 1991; Sharon *et al.* 1992) are the most frequent techniques used. Detection and quantification is achieved in most cases through the implementation of an ultraviolet detector. This device would detect the specific UV absorbance of the compound and

allow for its detection and separation from other components in the sample.

Use of *in vitro* Cultures to Measure Phytoalexins

The *in vitro* growth of cells in either suspension or callus cultures has been commonly used as a technique for the bioassay of phytoalexins (Latunde-Dada and Lucas 1985; Gustine *et al.* 1990; Wijnsma *et al.* 1985; Basse *et al.* 1992). *In vitro* techniques for phytoalexin extraction and measurement offer several advantages over intact plants. The main advantages are the ability to work with one or several similar cell types, and the ability to control environmental and nutritional variables (Miller *et al.* 1984). With the proper control of medium and environment, the same host-pathogen interaction is expressed *in vitro* as *in vivo* (Deaton *et al.* 1982; Helgeson *et al.* 1972). However, the relation between phytoalexin production *in vitro* and *in vivo* cannot always be determined because of the need to use separate and sometimes very different procedures to extract the same components and the presence of different chemical compounds *in vivo* (Duchesne 1993). Other reasons why cell cultures are used instead of intact plants or plant tissues are the ease of growth in tissue culture, reduction of pigments which may interfere with chromatographic measurement, the ability to preserve the plant from which it is derived, and the ease by which either elicitors or pathogens can be directly applied to the cells.

Several studies have shown that resistance in callus was associated with a colour change, in most cases a darkening of the callus tissue (Latunde-Dada and Lucas 1985; Miller *et al.* 1984). Darkened callus from resistant plants was also shown to have high

accumulations of phytoalexins (Latunde-Dada and Lucas 1985), so visual selection for resistance in calli could be used.

Mansonones

The mansonones are a family of sesquiterpenoid napthoquinones which were initially isolated from the heartwood of *Mansonia altissima*, a tree from west Africa which is used to make furniture (Bettolo *et al.* 1965; Tanaka *et al.* 1966). Bettolo (1965) isolated the mansonones in an attempt to identify the agents responsible for the irritative symptoms and heart troubles associated with workers involved in using the wood. Six compounds were initially extracted and designated as mansonones A to F. Subsequently, in other extractions using acetone, mansonones G and H were identified (Tanaka *et al.* 1966).

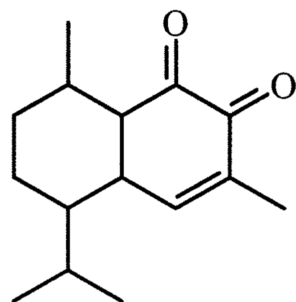
The first extraction of mansonones from *Ulmus* was accomplished by Elgersma and Overeem (1971) while they were attempting to discover if phenol oxidation was involved in the discoloration of the outer xylem after infection by *O. ulmi*. Mansonones E and F were isolated from the xylem of young branches of *Ulmus hollandica* 'Belgica' (Overeem and Elgersma 1970). Subsequently a total of six mansonones was identified and extracted from several different *Ulmus* species (Overeem and Elgersma 1970; Elgersma and Overeem 1971; Duchesne *et al.* 1986; Duchesne *et al.* 1985; Proctor and Smalley 1988; Jeng *et al.* 1983; Dumas *et al.* 1983). Extractions from infected *U. americana* wood has revealed six mansonones A, C, D, E, F and G (Fig 1.) (Duchesne *et al.* 1985; Dumas *et al.* 1983). These mansonones have a fungitoxic affect on *O. ulmi*

and *O. novo-ulmi*, and inhibit their growth and are therefore classified as elm phytoalexins (Dumas *et al.* 1986; Wu *et al.* 1989; Wu *et al.* 1985; Proctor *et al.* 1994).

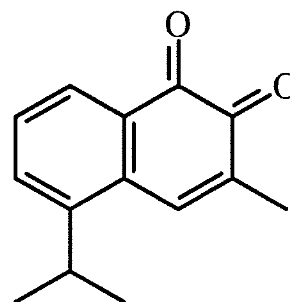
When mansonones were extracted from infected elm tissue, mansonones E and F were the two most important comprising 40.0% and 32.4% of the total fraction, respectively (Duchesne *et al.* 1985). The most fungitoxic of the mansonones is A followed by E (Dumas *et al.* 1986), although another study found mansonone E to be the most toxic (Wu *et al.* 1985).

Mansonones and Elm Resistance

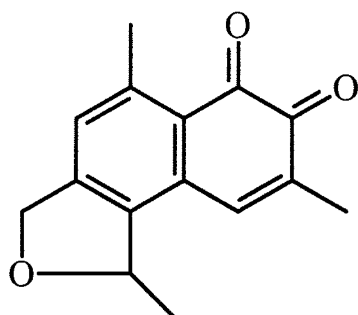
Elms which produce mansonones rapidly after infection are more resistant to DED. If mansonones are secreted quickly in the site of inoculation, the fungal growth rate will be dramatically reduced and the area of infection may be compartmentilised and the spread of the pathogen. The higher the concentration of mansonones at the infection site, the more toxic it is to the fungus. Mansonones extracted from infected xylem were in concentrations of 10 times higher than is required for significant reduction of *O. ulmi* growth (Proctor and Smalley 1988; Wu *et al.* 1989). Therefore the concentration of mansonones *in vivo* is great enough to arrest growth, but the question remains as to whether mansonones contact the fungus inside the plant. Some studies have found that, in resistant elm clones, the amount of mansonones accumulated is larger than in susceptible clones (Proctor and Smalley 1988). Mansonones produced in elm species with greater resistance to DED are more quickly accumulated resulting in a greater total concentration (Duchesne *et al.* 1986; Proctor and Smalley 1988; Szczegola *et al.* 1987).



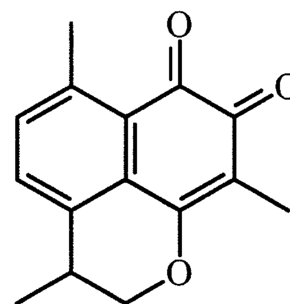
Mansonone A



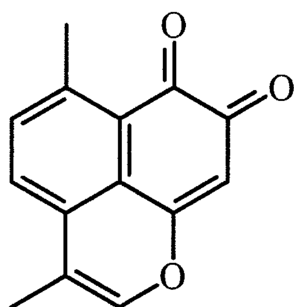
Mansonone C



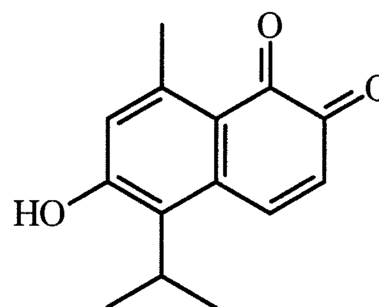
Mansonone D



Mansonone E



Mansonone F



Mansonone G

Figure 1 Chemical structures of Mansonones found in *U. americana*

The idea that mansonones are involved in DED resistance is more accepted since induced resistance in elms was documented (Hubbes and Jeng 1981; Jeng *et al.* 1983). Induced resistance is the acquired immunity to further infection by virulent strains of a pathogen following inoculation with an avirulent strain. Jeng *et al.* (1983) concluded that six compounds isolated from elms previously inoculated with a low virulence strain played a part in resistance. These compounds were thought to be phytoalexins but were not identified as such at that time. The resistance in *U. americana* was induced by initial inoculation with a non-aggressive strain of *O. ulmi*. Further studies have provided evidence that more mansonones are induced when elm is initially inoculated with a non-aggressive strain of *O. ulmi* (Duchesne *et al.* 1984; Duchesne *et al.* 1985). The evidence that non-aggressive, or low virulence, strains elicit more mansonones than aggressive ones could also provide an explanation as to why one strain is aggressive on elms while the other is non-aggressive. A strain with low virulence can be considered non-aggressive because the host reacts to an infection by this strain by producing mansonones. The mansonones do not allow the non-aggressive pathogen to flourish and thus it is not aggressive to the host. In contrast the aggressive strain does not elicit the mansonones and is able to spread in the host. It was then postulated that aggressiveness in *O. ulmi* was inversely correlated with mansonone accumulation (Duchesne *et al.* 1985; Duchesne 1993).

The use of mansonones as a tool for the selection of resistant elm is therefore possible. It is known that mansonones are associated with stained xylem tissues. Proctor and Smalley (1988) demonstrated that stained xylem had a 50 times greater concentration

of mansonones than did uninoculated xylem tissue. The staining of xylem between different elm individuals also varies (Green *et al.* 1985). Therefore, it could be assumed that total mansonone production would also vary between genotypes. Screening of large populations of elms for differences in mansonone production has not yet been accomplished. This data would be useful to identify genotypes that accumulate mansonones rapidly and in high concentrations for use in resistance selection.

In contrast to studies which show a relationship between mansonone production and DED resistance, several studies have provided evidence that mansonones have a relatively minor role, if any at all, in the resistance of elms to DED (Elgersma and Overeem 1971; Proctor *et al.* 1994; Proctor and Smalley 1990). Elgersma and Overeem (1971) observed no correlation of mansonone production to resistance because a resistant elm clone did not produce more mansonones than a susceptible elm. This finding has been questioned however. For example, when this research was done there was no distinction between aggressive and non-aggressive isolates of *O. ulmi* and there ability to elicit varying amounts of mansonones. Also, boiling alcohol which could denature mansonones was used in the extraction procedure, and mansonone content was expressed by number of cuttings rather than dry weight which lead to a non-precise concentration assessment (Hubbes 1988).

Although previous studies provided evidence that mansonones are responsible for the reduction of growth of *O. ulmi* (Wu *et al.* 1985; Wu *et al.* 1989), and are associated with resistance in elms in several other cases (Duchesne *et al.* 1986; Hubbes 1993), Proctor *et al.* (1994) recently demonstrated that tolerance to mansonone E is not required

for *O. novo-ulmi* to be virulent on *U. americana* and therefore discount the role of mansonones in elm resistance. However, to demonstrate this, it was necessary to generate mutants with low tolerance *in vitro* to mansonones E and F. These mutant strains, when inoculated into *U. americana*, exhibited wild type virulence (i.e., very virulent). This research is strongly based on the assumption that tolerance to mansonones *in vitro* and *in vivo* are similar. The environmental differences between agar plates and xylem tissue are great enough to pose a question to the validity of this study. Another question arises with respect to the mutants. Perhaps during mutation for mansonone tolerance other changes have also taken place, such as an interruption of the elicitation properties of the mutants. Proctor *et al.* (1994) state themselves that mansonones may at least have a limited role in resistance.

Toxicity of Mansonones

The toxicity of mansonones to *O. ulmi* has been clearly demonstrated. *O. ulmi* and *O. novo-ulmi* are both inhibited in their linear growth *in vitro* by mansonones (Dumas *et al.* 1986; Proctor *et al.* ,1994). The non-aggressive species, *O. ulmi*, shows the greatest variability in its sensitivity to mansonones. Dumas *et al.* (1986) found two strains varied from 91% to 23% inhibition. While mansonones inhibit linear growth of *O. ulmi* and *O. novo-ulmi*, they also inhibit a great range of plant pathogens, as is usually the case for phytoalexins, and are for the most part more effective in reducing linear growth of non-host pathogens (Proctor *et al.* 1994). Mansonone E was found to be the most effective in inhibiting dry weight accumulation of *O. ulmi* (Wu *et al.* 1989; Wu *et al.* 1985).

The cytological and physiological effects of mansonone E on *O. ulmi* are increased cellular ion leakage, and a reduction in respiration rate. Ultrastructural changes of fungal cells include membrane disruption, ribosomal aggregation, and the aggregation of electron dense material in the mitochondria (Wu *et al.* 1989). Plasmalemma breakage and separation from the cell wall along with eventual cytolysis leading to cell death was also observed in both species of *Ophiostoma* (Wu *et al.* 1985). These changes occur at much lower concentrations of mansonones than are extracted from infected xylem tissue. Therefore, if mansonones are at toxic concentrations at the site of fungal invasion, they could play a predominant role in disease resistance.

Elicitation of Mansonones

Since phytoalexins are elicited by compounds contained within the fungal cells attempts to isolate these specific elicitors of *O. ulmi* have been undertaken. Yang *et al.* (1989) noted that elicitation from cell wall extracts of *O. ulmi* induced higher amounts of mansonones than did cytoplasm and culture filtrate elicitors of the fungus. The age of the culture filtrate also affected its elicitation ability. As the culture aged from 2-4 days to 12-14 days, the elicitation ability of the filtrate increased. This could be due to structural changes of the elicitor over time making it more efficient at elicitation (Hubbes 1993). Elm species react differently to the same elicitor treatment indicating variation in the receptor system of the elms. The purification of the elicitor has revealed that a 21,000 dalton protein band is the main elicitor for mansonone production in the culture filtrate of *O. ulmi* (Hubbes 1993).

A hypothesis for phytoalexin accumulation was devised by Davis *et al.* (1986), who stated that phytoalexins are produced after cell death by surrounding healthy cells. This may apply to mansonones because, Yang *et al.* (1989) with abiotic elicitors showed that mansonones were only produced in those treatments where a portion of the cells died. Treatments in which all the cells died did not produce mansonones perhaps because they died so rapidly (Yang *et al.* 1989).

Tissue Culture and Mansonones

As in studies of phytoalexins in general, *in vitro* growth of nondifferentiated callus tissue is a valuable tool in mansonone studies. Szczegola *et al.* (1987) found that callus of elm was a valuable means of testing for mansonone production. Mansonone concentrations extracted from callus are similar to those extracted from infected wood (Duchesne *et al.* 1986; Yang *et al.* 1989), making callus culture a useful method to bioassay mansonone production in *Ulmus*. Mansonone F from callus of the resistant *U. pumila* accumulated quicker and also the total amount of mansonone F accumulated was greater (Szczegola *et al.* 1987). This was similar to observations for mansonone accumulation *in vivo* (Proctor and Smalley 1988).

Calli from different cell lines produced different amounts of mansonones, thereby showing that variation in mansonone production can be expressed in callus (Szczegola-Derkacz 1988). This is the only study to date which has investigated mansonone production in callus tissue of various genotypes. Using callus culture to measure mansonones is a versatile procedure in that experiments on elicitors and abiotic treatments

to elicit mansonones can be performed easily (Yang *et al.* 1989).

Extraction and Analysis of Mansonones

Mansonones are extracted from wood and callus using organic solvents. The most popular method is extraction using 80% ethanol (Dumas *et al.* 1983; Duchesne *et al.* 1985; Duchesne *et al.* 1986; Jeng *et al.* 1983; Overeem and Elgersma 1970; Dumas *et al.* 1983). Boiling ethanol was utilized for some of the first extractions but it was thought that this caused denaturation (Bell and Mace 1981; Overeem and Elgersma 1970). After mansonones have been extracted with ethanol they are then transferred to chloroform and concentrated or dried and taken up in another organic solvent for analysis.

Mansonones have mostly been isolated through thin layer chromatography (TLC). Small aliquots of extracts are spotted on the plates. After the plates have been developed mansonones are analyzed spectrometrically at 254nm (Dumas *et al.* 1983; Duchesne *et al.* 1985; Duchesne *et al.* 1986; Yang *et al.* 1989). Mansonones were initially identified when TLC plates were developed and regions on the plates which inhibited microbial growth were analyzed and mansonones extracted (Overeem and Elgersma 1970).

High performance liquid chromatography (HPLC) is a powerful tool in analysis. It is much more rapid, accurate and precise than TLC in many areas. The use of HPLC to isolate mansonones has not been used frequently. Proctor and Smalley (1988), in a published abstract, mentioned using HPLC to analyse mansonones quantitatively but no protocol appears to have ever been published. M. Dumas (pers. comm.) had initiated preliminary work on a HPLC system to identify and quantify mansonones but had not

perfected the system so that it could be used in mansonone detection studies. Thus, there is a need for the development of a HPLC protocol to analyse mansonones. This protocol could perhaps improve the knowledge of how mansonones are involved in resistance.

Chapter 1: Identification and Quantification of Mansonones from *Ulmus americana* L. by High Performance Liquid Chromatography (HPLC)

Introduction

The mansonones are a group of sesquiterpenoid naphthoquinones which possess phytoalexin activity within the genus *Ulmus*. These phytoalexins were first isolated from the heartwood of the *Mansonia altissima* Chev. (Bettolo *et al.* 1965). Six mansonones (A [$C_{15}H_{22}O_2$], C [$C_{15}H_{14}O_2$], D [$C_{15}H_{14}O_3$], E [$C_{15}H_{14}O_3$], F [$C_{15}H_{10}O_3$] and G [$C_{15}H_{13}O_2OH$]) (Fig. 1.0) have been isolated from *Ulmus americana* L. (Dumas *et al.* 1983). Mansonone production within elm tissue has been correlated to Dutch elm disease (DED) resistance (Duchesne *et al.* 1986; Jeng *et al.* 1983). Mansonones are induced in elm tissue as a result of elicitation by the DED fungi *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier. Studies have shown mansonones to be fungitoxic to both species of *Ophiostoma* and therefore play some role in the resistance of elm to DED (Dumas *et al.* 1986; Wu *et al.* 1989). In the past, mansonones have been quantified in elm tissue using thin layer chromatography (TLC) (Duchesne *et al.* 1986; Yang *et al.* 1989). Since mansonones are subject to rapid oxidation it is critical to analyse them as quickly as possible in the absence of oxygen. Since single high performance liquid chromatography (HPLC) run times can be quicker than TLC and sample runs are a one step process, it may be beneficial to use HPLC for mansonone analysis. The use of HPLC was initiated by Proctor and Smalley (1988), but not completely documented. Dumas, Canadian Forest Service, Sault Ste. Marie developed the technique further (M. Dumas, Pers. Comm.), to the point where it needed solvent programming, up to a 30 minute run time and variable wavelength detector. In order to test numerous samples, a shorter run time, fixed wavelength recorder and removal of the need for solvent

programming would be desirable. The objective of this study was to develop and document a HPLC method for analysis of mansonones E, F, and G from *U. americana* callus tissue. Such a method would be important since these isomers have been shown to be fungitoxic.

Materials and Methods

Chemicals

Solvents (acetonitrile and HPLC water) were obtained from Malinckrodt Paris, Kentucky, USA and J.T. Baker Phillipsburg, New Jersey, USA respectively. Samples were stored in 100% acetonitrile. Pure mansonone standards E, F, and G were supplied by M. Dumas, Canadian Forest Service, Great Lakes Forestry Institute, Sault Ste. Marie, Ontario.

HPLC Quantification of Mansonone Standards

A Waters Associates model M48 HPLC pump and a Waters Associates model 440 ultraviolet absorbance detector at 254 nm were used for mansonone analysis. A 20 µL sample was injected onto a Vydac reverse phase C_{18} 150 x 4.6 mm HPLC column operated at a flow rate of 1 ml/min.

Development of HPLC Technique

Several different solvent concentrations were tested using mansonone standards to see what effect they had on peak resolution and mansonone elution times.

Concentrations tested included 85% CH_3CN: 15% H_2O, 50% CH_3CN: 50% H_2O, and 30% CH_3CN: 70%H_2O. Equipment constraints allowed only a constant solvent concentration to be in use during a run. Solvent programming was not available and thus did not enter into the development of the technique.

Mansonone Standard Curve Development

Mansonones were diluted into several different concentrations. Mansonones E and F were diluted to 200, 160, 120, 80, 40, 20, 10, 5, and 2 µg/ml concentrations while mansonone G was diluted to 120, 96, 72, 48, 24, 12, 6, 3, 1.2 µg/ml. Mansonone G was in lower concentrations because it was produced in smaller amounts in elm tissue (Duchesne *et al.* 1985).

Known standard concentrations of mansonones were plotted against peak height. Linear regressions were obtained to determine the accuracy of the HPLC in the measurement of mansonones.

Mansonone Measurement in Callus Tissue

After the HPLC technique was developed, mansonones were measured within callus tissue of elm. Callus was grown, inoculated with *O. novo-ulmi* and then mansonones extracted as outlined below. Samples were run using the newly developed HPLC protocol, peak heights measured and mansonone concentration calculated.

Callus culture

Ulmus americana seed was collected in the spring of 1993 from several locations throughout Manitoba, Canada, as part of a larger study (Chapters 2 and 3). Seed was stored at -20°C. In 1993, seed was germinated in a soilless medium (Metro Mix™). Seven to ten days after emergence both cotyledons were removed from the seedlings for culture. Cotyledons were then sterilized by placing them in 20% hypochlorite with 0.1% Tween 20™ as a wetting agent for 20 minutes and cotyledons were then rinsed three times for five minutes each in autoclaved deionized water. The cotyledons were cut in half and plated onto modified B5 medium (Appendix A) (Gamborg *et al.*1968) with 1.5 µg/ml 2,4-D according to the procedure of Yang *et al.* (1989). Calli were grown in the dark at 27°C and subcultured once onto the same medium.

Inoculation of callus

Calli were inoculated with 1 ml of $1x10^6$ spores/mL^{-1} *O. novo-ulmi* strain CESS 16K spore solution in Takai and Richards media (Appendix B). Calli were allowed to incubate for 24 hours, then each piece of callus was wrapped in aluminum foil and frozen in liquid nitrogen for approximately 2 minutes to arrest elicitation. Calli were then stored at -70°C until samples were prepared for mansonone extraction.

Extraction of mansonones

Calli were weighed and mansonones extracted by the methods described in Duchesne *et al.* (1986). Treated calli were homogenized in 80% ethanol (10:1

ethanol:calli [v/w]) with a Polytron homogenizer (Kinematica GmbH, Switzerland), for 2 minutes. The slurry was then transferred to a 125 mL erlenmeyer flask and placed on a rotary shaker at 120 rpm for 18 h. Samples were filtered through Whatman no.1 filter paper. Ethanol extracts were evaporated to 10 mL on a rotary evaporator and partitioned with chloroform (2.5:1 chloroform:extract, v/w) in a 125 mL separatory funnel. The chloroform extract was evaporated to dryness on a rotary evaporator at 20°C. Residue was taken up with 2 mL of acetonitrile for HPLC analysis. Samples were stored at minus 70°C. Prior to HPLC analysis samples were filtered through a 0.45μ syringe filter.

Results

HPLC System

To develop a protocol to measure mansonones using HPLC, the solvent system of the HPLC was modified several times to find the optimal solvent concentration. As the concentration of acetonitrile increased the mansonones eluted more quickly and mansonone peaks appeared closer together. When peaks are too close the resolution between peaks is reduced. As the concentration of water was increased the compounds eluted more slowly but there was better peak resolution and separation (Table 1.1). The concentration of 70% water: 30% acetonitrile was found to be the most favourable to use in the HPLC analysis of mansonones. This concentration of solvents gave the best peak resolution as well as a reasonable elution time for all three mansonones.

Table 1.1 Effects of varying solvent concentration on mansonone elution characteristics.

Solvent Concentration $CH_3CN:H_2O$	Elution Time of Mansonones E, F, and G (Minutes)	Resolution of Peaks
85:15	2-3	No Resolution
50:50	4-6	No Resolution
30:70	12, 10, 14	Good Peak Resolution

Using the newly developed solvent system and HPLC equipment, pure mansonone standards were used in several different concentrations to produce standard curves (Figs. 1.1 to 1.3). The r^2 value for all three standards was 0.99. The standard curves were then used to estimate mansonone concentration within the callus samples.

The fixed 254nm UV wavelength detector was sufficient for detecting the three mansonones. All three mansonones had absorbance peaks in their UV spectra near 254 nm (Table 1.2). This indicates that if a fixed 254 nm is used it will detect all of the mansonones because each of them have some absorbance at this wavelength.

Table 1.2 UV spectrum for Mansonones E, F, and G.

Mansonone	UV spectrum	Log ε
E	445, 368, 264, 220 nm	3.01, 3.01, 4.14, 3.87
F	550, 335, 255, 235 nm	3.52, 3.46, - , 4.23
G	233, 288, 328 nm	Not Reported

UV spectral data adapted from (Chen *et al.* 1990) and (Tanaka *et al.* 1966).

Measurement of Samples

The HPLC protocol was efficient in the measurement of mansonone extractions from callus tissue. Examples of the range of mansonone production measured by the HPLC system are shown in Table 1.3. These data were obtained as part of a larger study (see chapter 2).

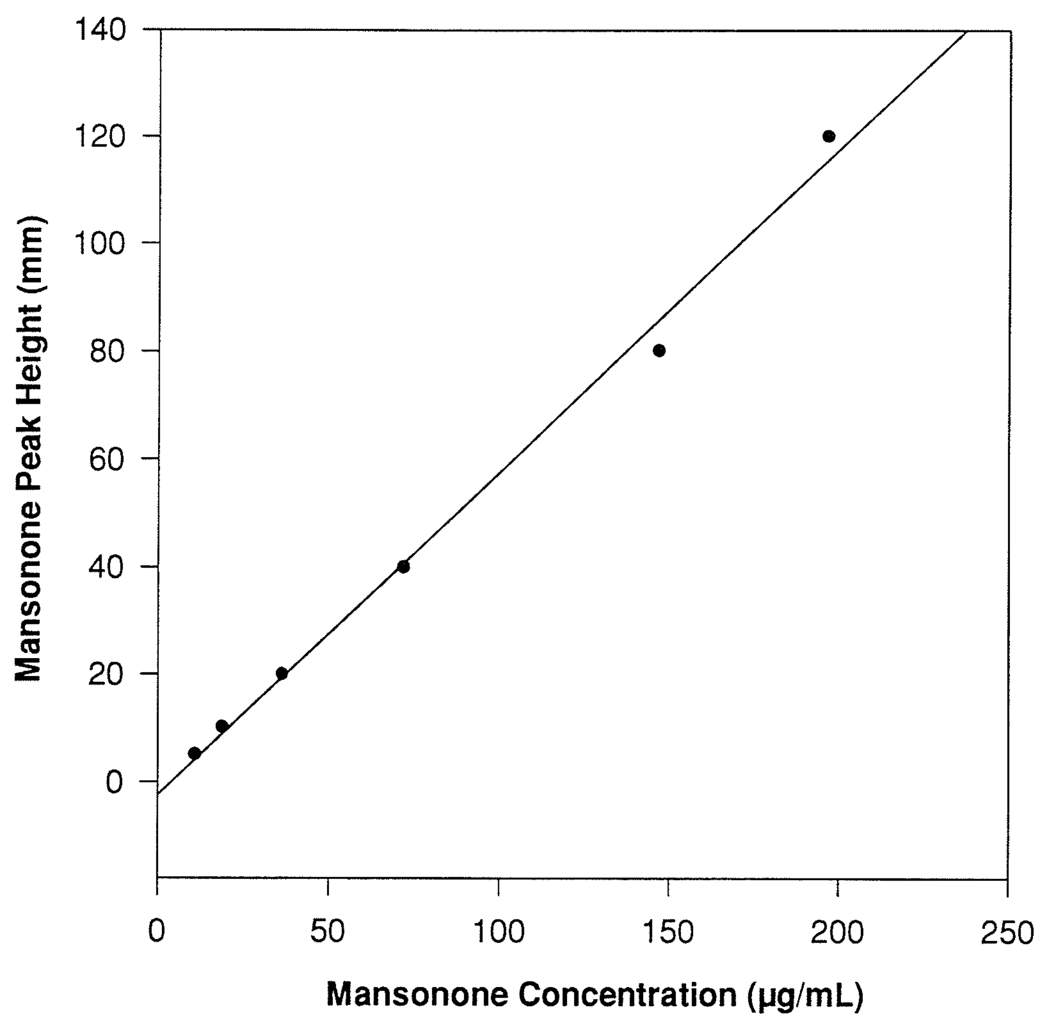


Fig. 1.1 Standard curve for mansonone E derived through HPLC analysis. Y = -2.38 + 0.60X. Where Y = mansonone peak height(mm) and X = mansonone concentration (µg/mL). r^2 = 0.99.

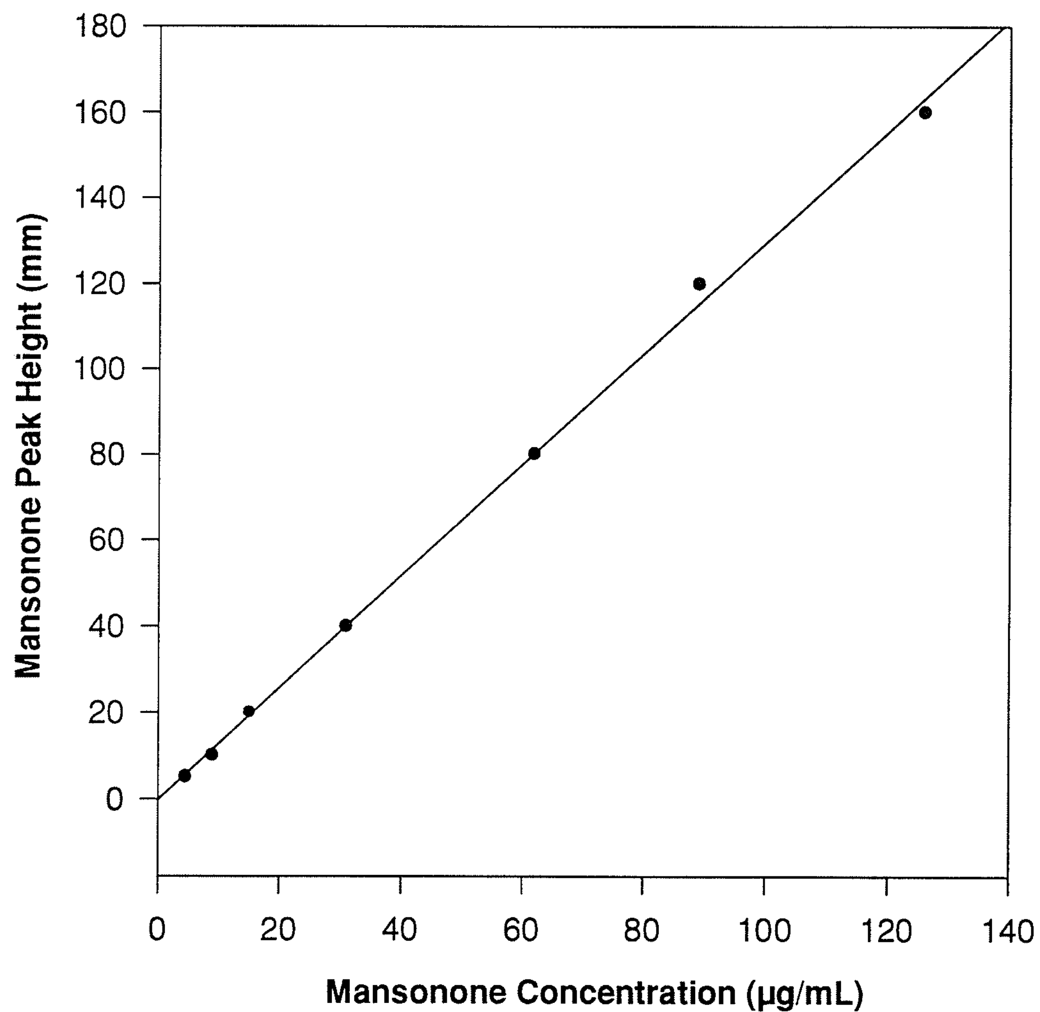


Fig. 1.2 Standard curve for mansonone F derived through HPLC analysis. Y = -0.21 + 1.30X. Where Y = mansonone peak height (mm) and X = mansonone concentration (μg/mL). r^2 = 0.99.

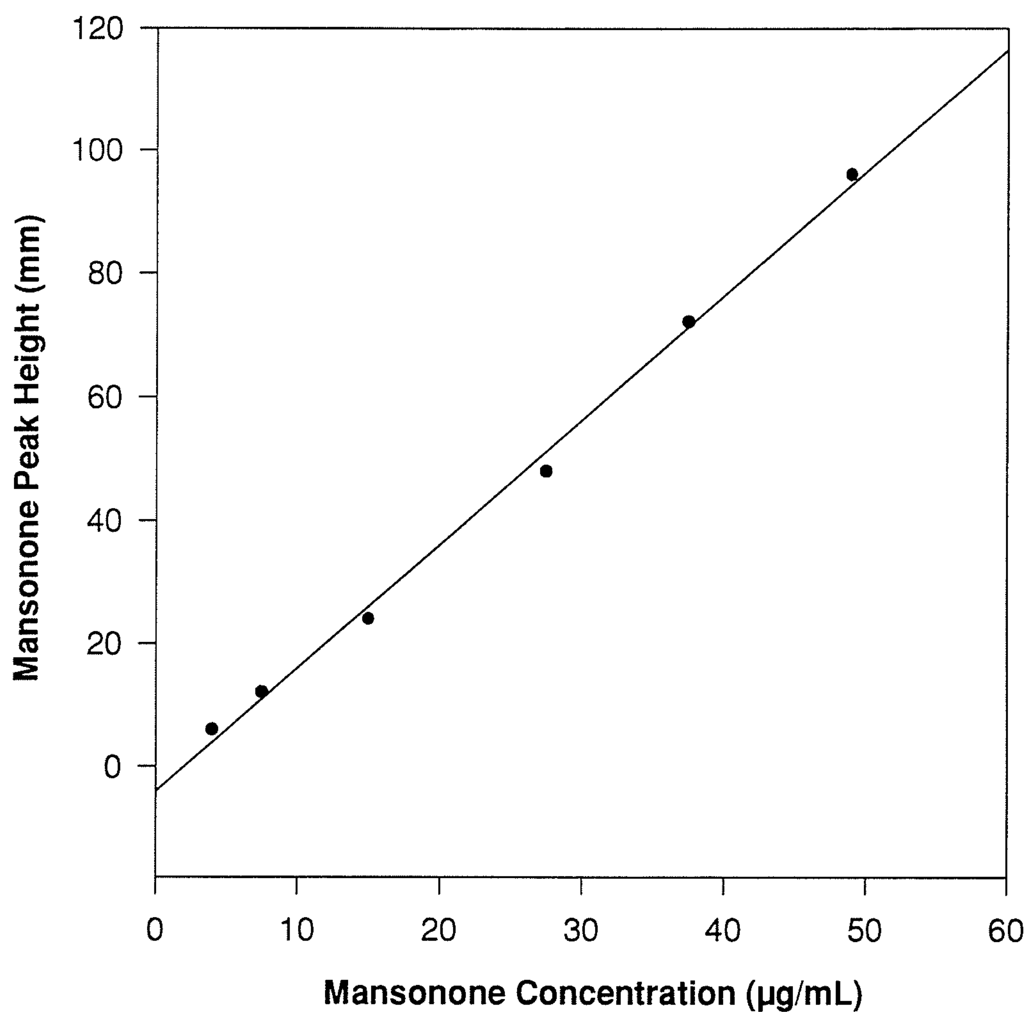


Fig 1.3 Standard curve for mansonone G derived through HPLC analysis. Y = -4.06 + 2.00X. Where Y = mansonone peak height (mm) and X = mansonone concentration (µg/mL). r^2 = 0.99.

Table 1.3 Mansonone concentrations (E, F, and G) for sample calli from progeny of various trees.

Genotype	Mansonone E µg/g	Mansonone F µg/g	Mansonone G µg/g
Lort-3 22	16.75	1.69	3.32
Beau-1 13	3.91	.06	2.54
Stan-2 24	13.45	2.13	1.14
Stan-4 8	10.94	2.78	7.96
Selk-4 1	17.71	2.23	9.22
Beau-14 8	1.13	0.05	0.23
Wpg-9 24	0.85	0.66	0
Skin-M 9	43.53	10.26	2.47

Note: Genotypes were selected from a wild population and represent a sample of the entire group.

Discussion

Preliminary HPLC analytical work on the measurement of mansonones was carried out by M. Dumas (pers. comm.). In the present study, the method has been simplified so that HPLC with a fixed wavelength detector can be used. Moreover, the need for solvent programming has been eliminated. This means there is no need to change the concentration of the solvent during the sample run resulting in a less complicated method using a single solvent concentration. The solvent concentration which gave the best peak resolution in the shortest elution time was 30% acetonitrile: 70% water (Table 1.1). Because a large number of samples were to be analysed as part of a larger study (see chapter 2), there was a need for a quick sample run time. The run time for a sample was found to be approximately 15 minutes. This compares to over 20 minutes for the HPLC

protocol used by Dumas. When screening hundreds of samples, this is a significant increase in efficiency.

The HPLC protocol developed in this study is rapid and efficient at measuring mansonone production in callus tissue. The mansonone concentrations shown in Table 1.3 are a sample of a large group of genotypes. The ability of the method to measure mansonones over such a broad range of concentrations proves its usefulness for screening large numbers of variable samples.

The ability to identify and quantify mansonones through the use of HPLC has provided an alternate to TLC. The mansonone concentrations measured by HPLC from callus tissue were similar to those measured by TLC from callus in other studies (Yang *et al.* 1989). HPLC can easily be automated and thus numerous samples could be analysed without the need for constant handling. When numerous samples are to be analysed by TLC it requires constant human attention to move the plates from station to station (Poole and Poole 1991). To conclude, the system of analysis presented in this study allows for the efficient analysis of a large number of mansonone samples. Screening vast numbers of elm for mansonone response could make the selection process for elms resistant to DED more rapid.

Chapter 2: Genotypes of *Ulmus americana* from Manitoba tested for differences in mansonone production and vascular staining.

Introduction

Mansonones are phytoalexins found in the genus *Ulmus*. Mansonones have been shown to be fungitoxic to *Ophiostoma ulmi*, the cause of DED (Wu *et al.* 1985; Proctor *et al.* 1994; Wu *et al.* 1989). The production of mansonones within elm has been linked to a part of the resistance mechanism (Duchesne 1993). The American elm is very susceptible to DED. Even though mansonones have been associated with increased resistance there have been no large scale tests to observe any genotypic differences among elms in their production. In a series of experiments by Szczegola-Derkacz (1988) it was shown that in a small number of samples there were differences in the production of mansonones. A large scale sample of native trees would provide information on the possible use of mansonones as a selection criteria for resistance in *Ulmus americana.*

Other indicators of resistance, such as vascular staining, may also be beneficial in a screening process (Green *et al.* 1985; Sinclair and Larsen 1980). Vascular staining is a symptom of DED where the internal vascular tissue has a brown streaked appearance. The stained area is usually associated with the extent of fungal invasion within the vascular tissue. If mansonone levels could be linked to symptom expression it would support the use of mansonone measurement as a screening tool for tolerance to DED within young seedlings.

Resistance in trees to fungal invasions can be variable, and several levels of resistance can occur within a species (Bell and Mace 1981). In some instances, a tree may react to an invasion and kill the invading agent while in others the defense reaction could be sufficient to prevent damage the infecting agent can cause. A plant in the

second case is considered to be tolerant to the invading agent, that is, it reduces the damage the agent causes but does not kill the agent (Clapper 1952). This type of a reaction in some cases is favoured over the resistant type in which the pathogen is killed. The reason why such a reaction is favoured is because some plants tolerate the disease and suffer no appreciable damage (Clapper 1952). Also the selection pressure on the pathogen is reduced for a tolerant reaction. Therefore a level of mansonone production and other host defense reactions in an elm would be beneficial at a level where the tree is tolerant rather than resistant.

Mansonones can be produced within elm callus tissue (Duchesne *et al.* 1986; Szczegola *et al.* 1987). The use of callus tissue has benefits over the use of actual stem tissue: these include the ease of uniform distribution of the fungal spores on the callus, the avoidance of destructive sampling of the actual tree, the ease of mansonone extraction, and the homogeneity of cell type (Miller *et al.* 1984). Moreover, the production of phytoalexins *in vitro* is often related to levels in the actual plant (Deaton *et al.* 1982; Helgeson *et al.* 1972), and therefore may provide a reliable and easily obtainable indicator of mansonone production potential.

The DED fungus has both an aggressive and a non-aggressive species. The non-aggressive species (*Ophiostoma ulmi*) is not as virulent to the elm as the aggressive species (*Ophiostoma novo-ulmi*) (Brasier 1991). Some of the strains of the non-aggressive species can be controlled by the elm's resistance mechanism but strains from the aggressive species will almost always kill the tree. Although *Ophiostoma novo-ulmi* elicits less mansonone production than *O. ulmi* (Yang *et al.* 1989), it is best suited for use

in screening experiments because it would be most beneficial to know the mansonone production in response to the most virulent species.

Using progeny from several populations of *Ulmus americana* located within Manitoba, the objectives of this study were to investigate differences in *in vitro* mansonone production and vascular staining among genotypes, individual trees within populations, and populations. Another objective of this research was to compare staining and mansonone production amounts among progeny from the same parents and populations to analyse relations between the two possible indicators.

Materials and Methods

Population Selection (collection sites)

Six separate populations of *U. americana* were identified in southern Manitoba. Population names correspond to either towns, cities or highways in the immediate area: Winnipeg, Lorette, St. Anne, Beausejour, Selkirk and Henderson.

Criteria used in the selection of populations were that each of the populations must have had previous infestations of DED. All populations had severe previous DED except for Winnipeg, where control efforts have lessened the impact of the disease. Locations with high incidence of DED were used to ensure that initial selection of weak genotypes by the fungus would increase the probability of selecting parent trees with a higher degree of resistance.

Parent Tree Selection

Within each population 15 parent trees were selected. Parent trees were chosen if they were reproductively mature, had vigorous and healthy growth and if crown form was typical of the species. Only 11 such parents were present within the Selkirk population.

Several different elm species and selections were included in the study for comparison. These were American elm selections *U. americana* cv Skinner's Upright and *U. americana* cv L'Assumption as well as *Ulmus* cv *Sapporro* Autumn Gold, *U. davidiana* var japonica and *U. pumila*. Seed from these selections were supplied by C.G. Davidson, Agriculture and Agri-Food Canada, Morden, Manitoba.

Seed (fruit) Collection

Fruit was harvested from each parent tree in the spring of 1993, when fruit was mature and still adhering to the crown. Pole pruners were used to remove upper crown branches bearing the fruit. Fruit was stripped from the branches and placed in paper bags. The Winnipeg population was harvested directly from branches utilizing a high-up truck.

The fruit was dried in the greenhouse to a moisture level of approximately 9%. It was then packed in air tight containers and stored at -18°C.

Seed Germination and Seedling Growth

Fruit was planted in Spencer Lamaire root trainers containing Metro mix™, a

soilless medium. A total of 96 containers per parent tree were planted in the greenhouse in early summer. Seedlings were watered and fertilized regularly with NPK (20-20-20). In late summer all seedlings were moved out of the greenhouse into cold frames, to begin acclimation.

Mansonone Production Determination

Using the material selected from several different populations throughout Manitoba, two experiments based on mansonone production were designed. The first experiment was designed to address the problem of genotypic differences among progeny in the production of mansonones. The second experiment investigated the differences in mansonones production among populations and parent trees within populations and to a lesser extent genotypic differences as well.

Replicated analysis of mansonone production by genotypes

Two cotyledons from 12 seedlings (population Beausejour) were cultured onto 6 petri plates. Cotyledons were removed from *U. americana* seedlings seven days after emergence for use in callus production. Callus was grown and inoculated as described in chapter 1.

The cotyledons of each of two genotypes were placed on each plate. Two pieces of callus were produced per genotype tested. Each piece was inoculated and measured for mansonone production as outlined in Chapter 1. A total of 12 genotypes was tested.

Mansonone production within populations and trees within populations

Five trees from each of the 6 populations were chosen for mansonone analysis. Twenty four progeny from each of the parents was used including 24 progeny from each of the 6 selections and species. Therefore, a total of 864 different genotypes was plated for callus production. Through losses due to infection and little or no callus production, there were only 235 samples left. There was no replication of progeny in this experiment.

Callus Tissue Production and Inoculation With *Ophiostoma novo-ulmi*

At 7-10 days after emergence, the cotyledons of the seedlings were removed and cultured for mansonone production experiments. The optimum timing of cotyledon removal was determined experimentally and is discussed in Appendix D. Callus was produced under the same conditions as described in chapter 1. The seedlings which had their cotyledons removed were planted at Carman, Manitoba to be used in future research. Calli were inoculated with fungal spores as described in chapter 1. Control calli were inoculated with sterilized fungal growth medium without *O. novo-ulmi*.

Inoculation of Seedlings With *Ophiostoma novo-ulmi*

Two studies were initiated to investigate the reaction of seedlings to inoculations with *O. novo-ulmi*. The two studies involved seedlings selected from the original six populations of elm within Manitoba. In one study, only visual symptoms of DED were recorded while in the second both visual symptoms and extent of vascular staining were measured.

Establishment of seedlings at Carman site

In the autumn of 1993, seedlings used for callus production were planted at the University of Manitoba Research Station at Carman, Manitoba, 75 km southwest of Winnipeg. Seedlings were hand planted into 4 rows which contained 6 progeny from each parent tree. Groups of six were randomized within rows. Spacing between plants was 25 cm. Trees were fertilized once with 27-14-0 at 1.5 kg pure N/100m^2. Weeds were controlled by cultivation. Damage from deer grazing was reduced by the use of animal repellents.

Establishment of seedlings at Bird's Hill site

The vascular staining experiment involved a total of 24 progeny from all the parents within the populations, plus 24 progeny from each of the selections or species. A total of 2208 seedlings were to be analysed for extent of vascular staining but due to field losses only 1775 seedlings were analysed. In the spring of 1994 the 24 progeny from each parent tree were planted at the provincial tree nursery at of Bird's Hill Provincial Park, 20 km north of Winnipeg. Progeny were divided into three blocks each block containing 8 seedlings per parent. A randomized complete block design was utilized. Seedlings were all hand planted at a 25 cm spacing.

The remaining seedlings from each parent which were not used in either the mansonone or staining experiments were planted out in a reserve block at 25 cm spacing for future research. Weed and pest control were similar to the Carman field trial.

Ophiostoma novo-ulmi culture and inoculation technique

O. novo-ulmi strain CESS 16K was supplied by W.C. Richards, Forestry Canada, Sault Ste. Marie, on petri plates of PDA (potato dextrose agar). In order to prepare spore suspensions, 3 discs 0.5 cm in diameter were removed from the plates using a corkborer. The discs were placed in 125 mL erlenmeyer flasks containing 50 mL of Takai and Richards medium (Appendix B). Flasks were placed on a rotary shaker at 120 rpm. After 72 h the fungal culture was filtered through 2 layers of cheesecloth to remove mycelium (Yang *et al.* 1989). Spore concentrations were determined using a haemacytometer.

Seedlings at both sites were inoculated in the spring of their second year of growth. Timing of inoculations was based on the requirement of active growth of stems. Approximately 2-3 cm from ground level a syringe was used to wound the main stem of the seedling. The wound penetrated through the bark into the wood, ensuring a route for spores to enter into the xylem flow. A generous amount (0.25-0.5 mL) of spore suspension, at a concentration of 1×10^7 spores/mL of water, was injected into the seedling and then the wound was wrapped with masking tape (Green *et al.* 1985).

Seedling symptom measurement

Visual symptoms of DED were recorded at both inoculation trials. Seedlings at the Bird's Hill site were harvested approximately 100 days after inoculation for analyses of vascular staining, by pruning them to soil level. Each plot of seedlings was placed in a separate plastic bag and labelled. Bags were stored at -15°C until dissections were

performed.

The total length of vascular staining acropetally and basipetally was measured on the inoculated seedlings. A scalpel was used to excise the bark and reveal the extent of staining. Percent staining was calculated as the percent of the stem above soil level which was stained.

Statistical Analysis

In replicated analysis of genotypic differences in mansonone production a one way analysis of variance (ANOVA) was performed on the data for each pair of calli from each of the 12 genotypes. A nested ANOVA was performed on the large data set resulting from the population and trees within population experiment. The model tested population and parent trees nested within populations. Another ANOVA was performed on the wild genotypes and elm selections and species. A relationship between increasing callus weight and decreasing mansonone production was detected and tested using regression analysis. When the mansonone production was log transformed it increased the linear relationship. Based on this analysis, the log of weight of callus was used as a covariate in the analysis of mansonone production. A correlation test was used to analyse the relationship between the extent of vascular staining and mansonone production among similar parents and populations. ANOVA tables for all the tests are presented in Appendix C.

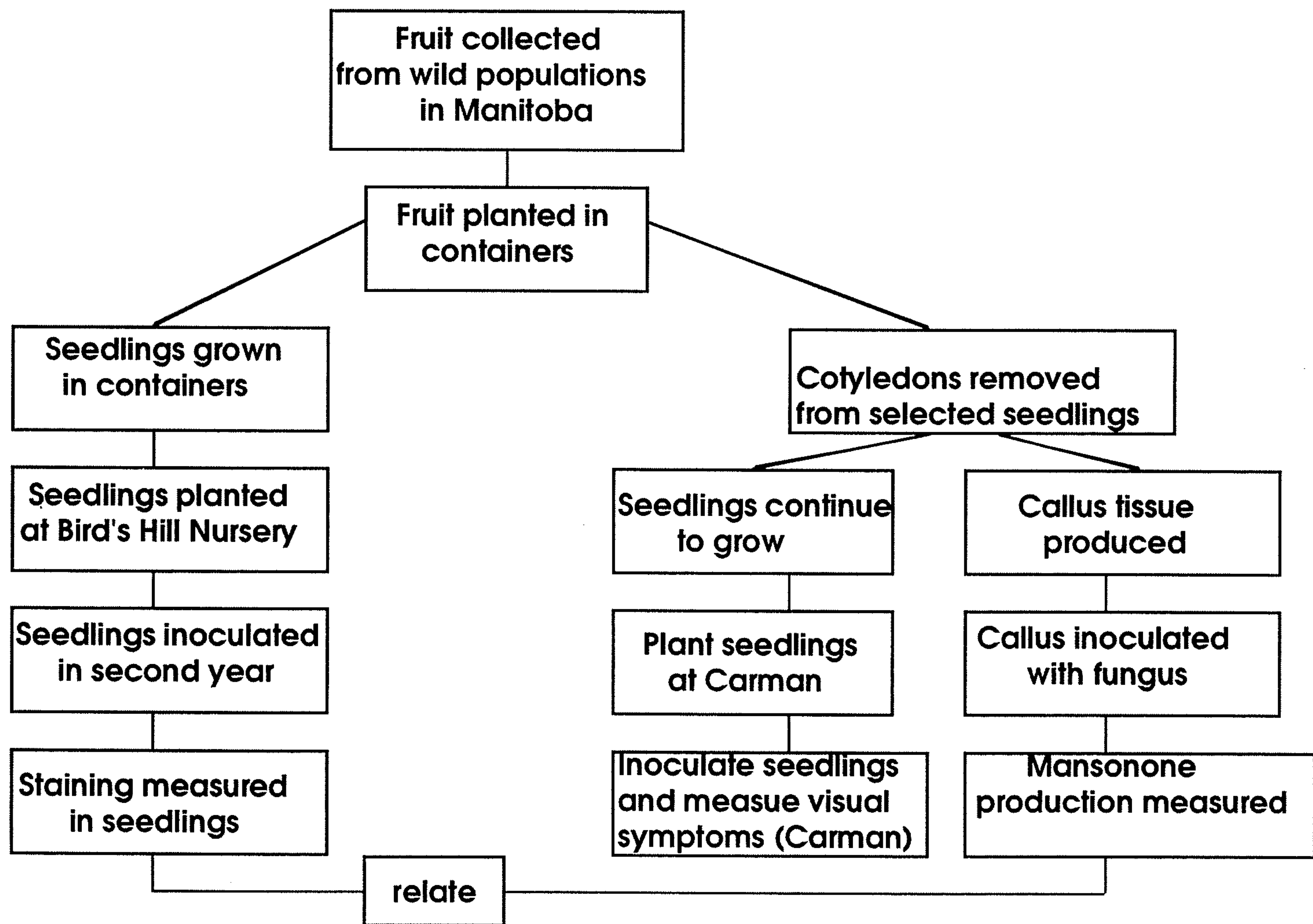


Fig. 2.1 Flow diagram showing expected steps in analysing elm material.

Results

Genotype Variation in Mansonone Production

A one way analysis of variance test revealed that differences in mansonone production were significant among the genotypes (P = 0.01). Differences among genotypes were greater than differences within genotypes (Fig. 2.2). Genotypes 1, 5, and 8 had similar production of mansonones for each of callus while 2, 6, and 11 varied considerably between calli (Fig. 2.2). Though in some cases the variation within a genotype was great, the variation between genotypes was still larger.

Variation in mansonone production among progeny from the same tree was quite large (Figs. 2.3 to 2.5). This large variation is represented by the large standard error for many of the parent trees tested for mansonone production (Table 2.1). Standard errors for the trees represented in Figs. 2.3 to 2.5 were quite high and many of the other trees had standard errors of that magnitude (Table 2.1).

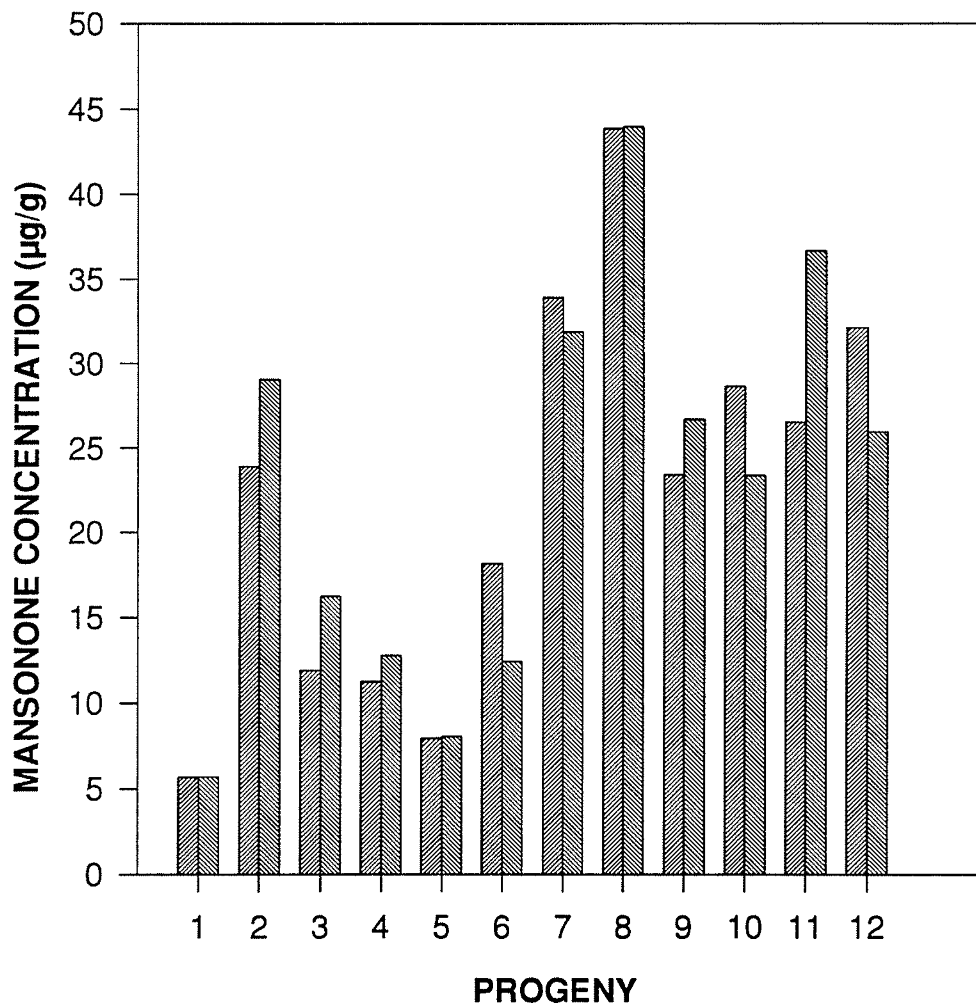


Fig. 2.2 Mansonone levels of progeny of 12 parent trees from population Beausejour. Two pieces of callus tested per progeny.

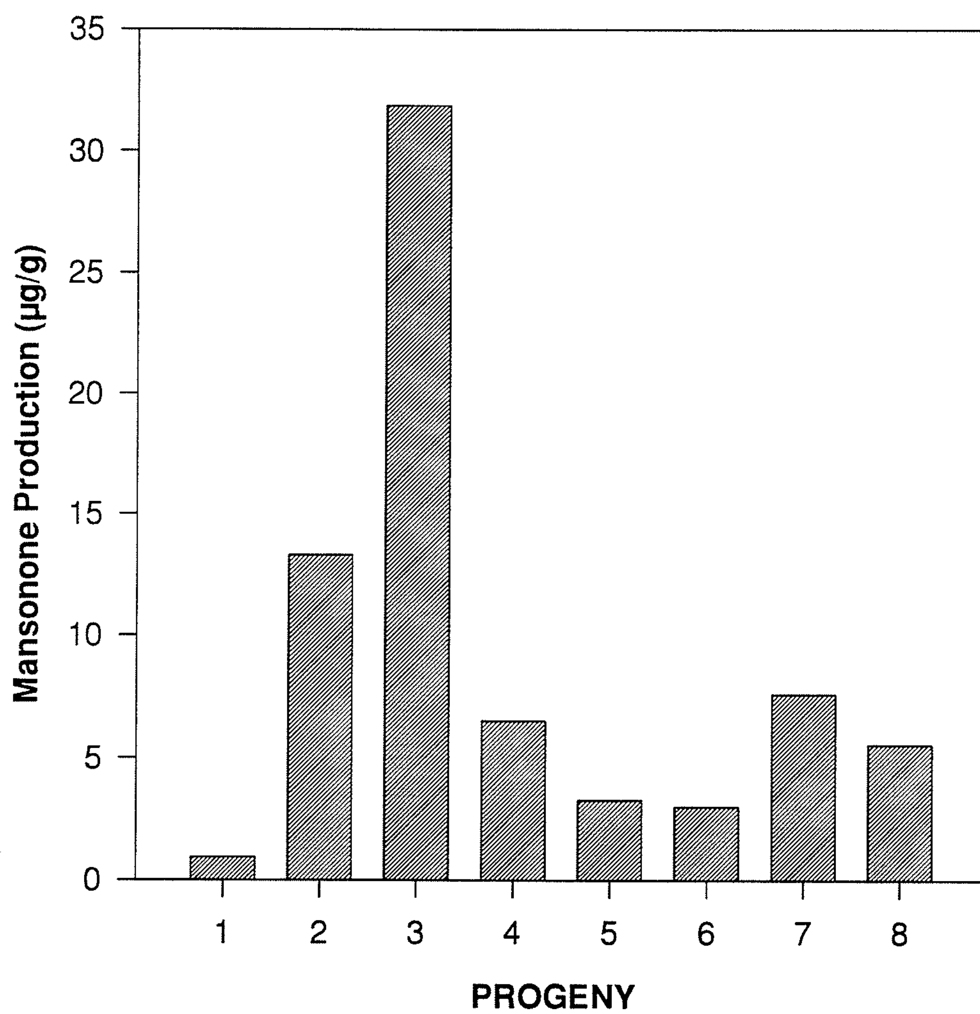


Fig. 2.3 Mansonone levels in progeny from a single tree in the Beausejour population (Beau-1).

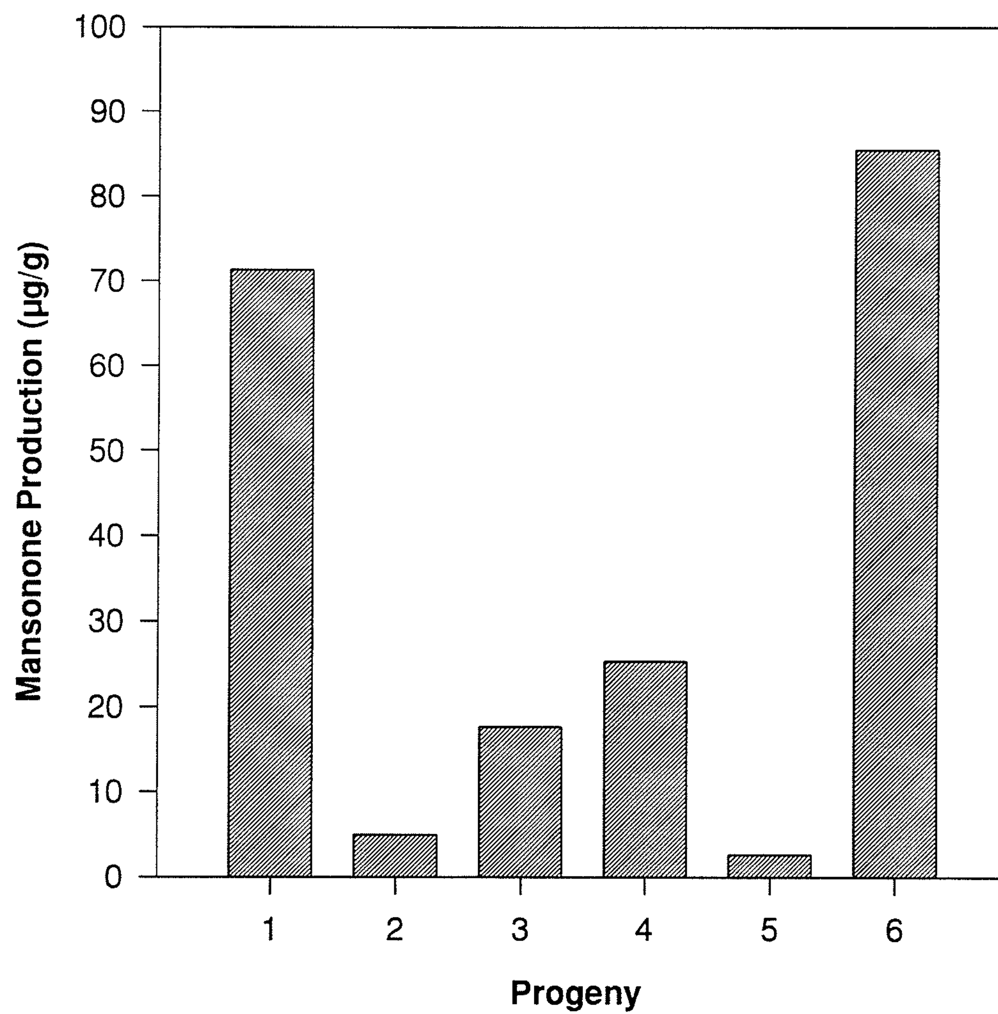


Fig. 2.4 Mansonone levels in progeny from a single tree in the Henderson Highway population (Hend-9).

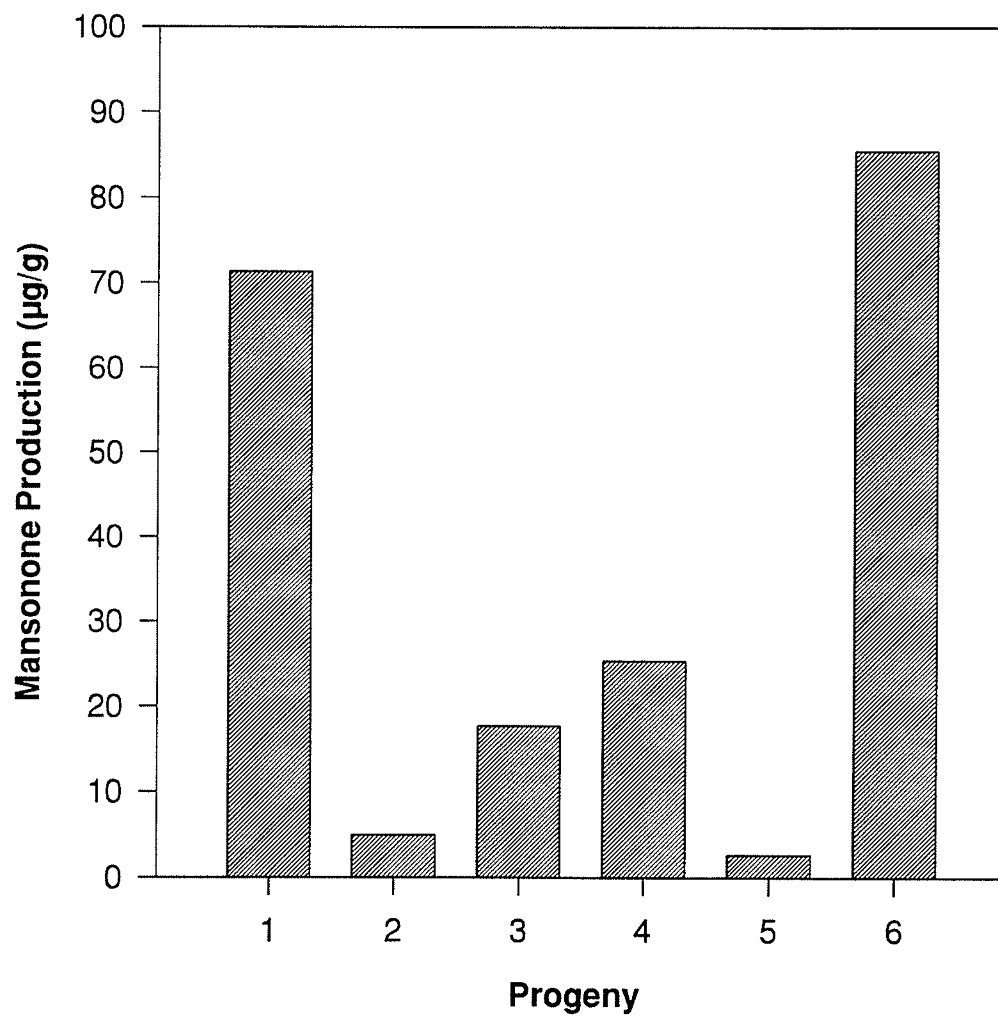


Fig. 2.5 Mansonone levels in progeny from a single tree in the Winnipeg population (Wpg-15).

Mansonone Production Among Populations and Parent Trees Within Populations

No significant differences in mansonone production were found among populations (P = 0.11) (Fig. 2.6). The mean mansonone values for populations did vary but the standard error for the population means were large resulting in a lack of significance. The lowest population mean was from the Henderson highway population and the highest means are St. Anne and Winnipeg populations.

Significant differences in mansonone production were found among parents within a population (P = 0.0006) (Table 2.1). The mean production of mansonones varied quite dramatically from one parent to the next. Mean mansonone production varied from a low of 2.03 µg/g to a high of 34.53 µg/g (Table 2.1).

A casual observation of the mansonone production data revealed a relationship between mansonone production and callus weight. It was noticed that as callus weight increased mansonone production dropped. Thus, a correlation between mansonone production and callus weight was evident (Fig. 2.7). However this relationship was not linear and the mansonone production data were log transformed to linearize them (Fig. 2.8). The log transformed weight of the callus was then used as a covariate in the tests for mansonone production, and even though the P value decreased slightly the test was still highly significant P = 0.0006 (Appendix C). An outlier was removed from the callus weight and mansonone production comparison. An outlier was extremely high for mansonone (246.04 µg/g) and skewed the data dramatically and was omitted from the analysis. This does not discount the biological importance of such a sample in selecting for tolerance.

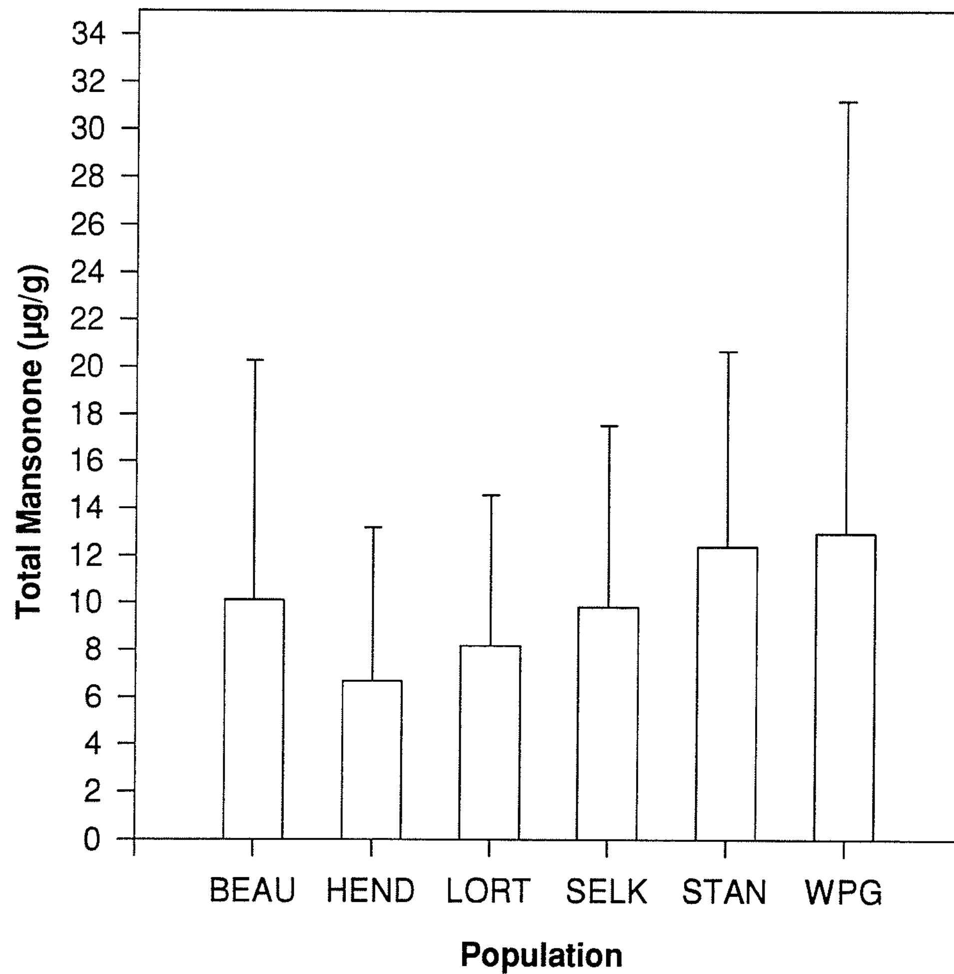


Fig. 2.6 Unadjusted mean mansonone levels (mansonones E, F, and G combined) for six populations of *Ulmus americana* within Manitoba. Means were not significantly different based on ANCOVA with callus weight as the covariate, P = 0.11.

Table 2.1 Unadjusted mean mansonone production (mansonones E, F, and G combined) for progeny from individual trees within populations.

Population	Tree	N	Mean	Std. Err.
Beausejour	1	8	9.00	9.33
	2	3	5.47	5.41
	3	4	6.60	0.90
	4	1	18.98	0
	5	12	12.50	12.32
Henderson	1	9	9.98	7.91
	2	2	2.03	0.67
	3	7	7.08	7.41
	4	11	3.41	1.38
	5	4	9.84	5.75
Lorette	1	9	13.46	8.05
	2	2	4.40	2.43
	3	2	4.67	3.14
	4	10	5.28	3.1
	5	4	7.21	1.07
Selkirk	1	6	14.85	7.53
	2	8	6.94	4.25
	3	4	7.97	9.44
St. Anne	1	6	10.22	3.06
	2	8	19.07	9.35
	3	4	4.83	1.9
	4	5	13.11	6.82
	5	2	5.48	1.65
Winnipeg	1	5	16.73	6.64
	2	7	5.52	3.61
	3	3	6.43	3.13
	4	11	5.92	4.24
	5	6	34.53	32.19

Note: Differences between populations not significant ($P = 0.11$).
Differences between trees significant at $P = 0.0006$.

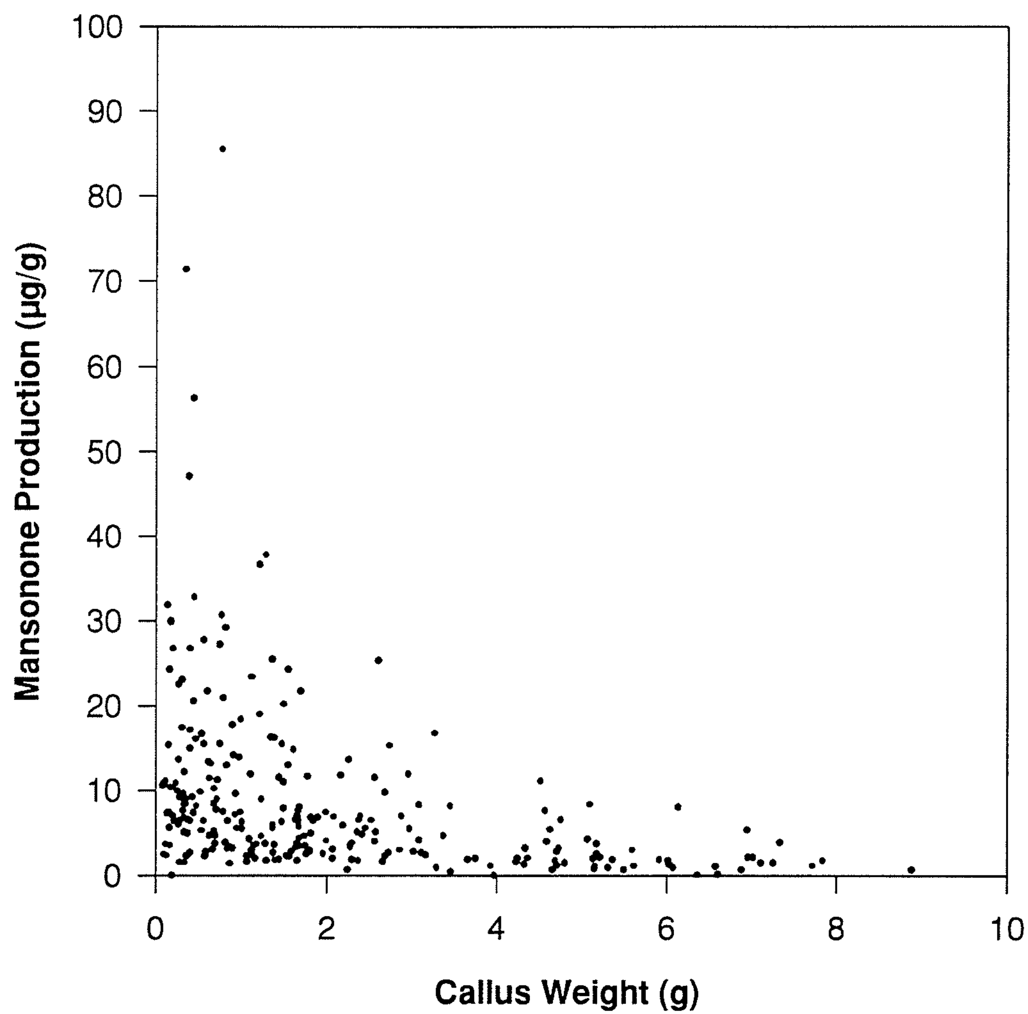


Fig. 2.7 Relationship between mansonone production and callus weight for all samples analysed for mansonone production.

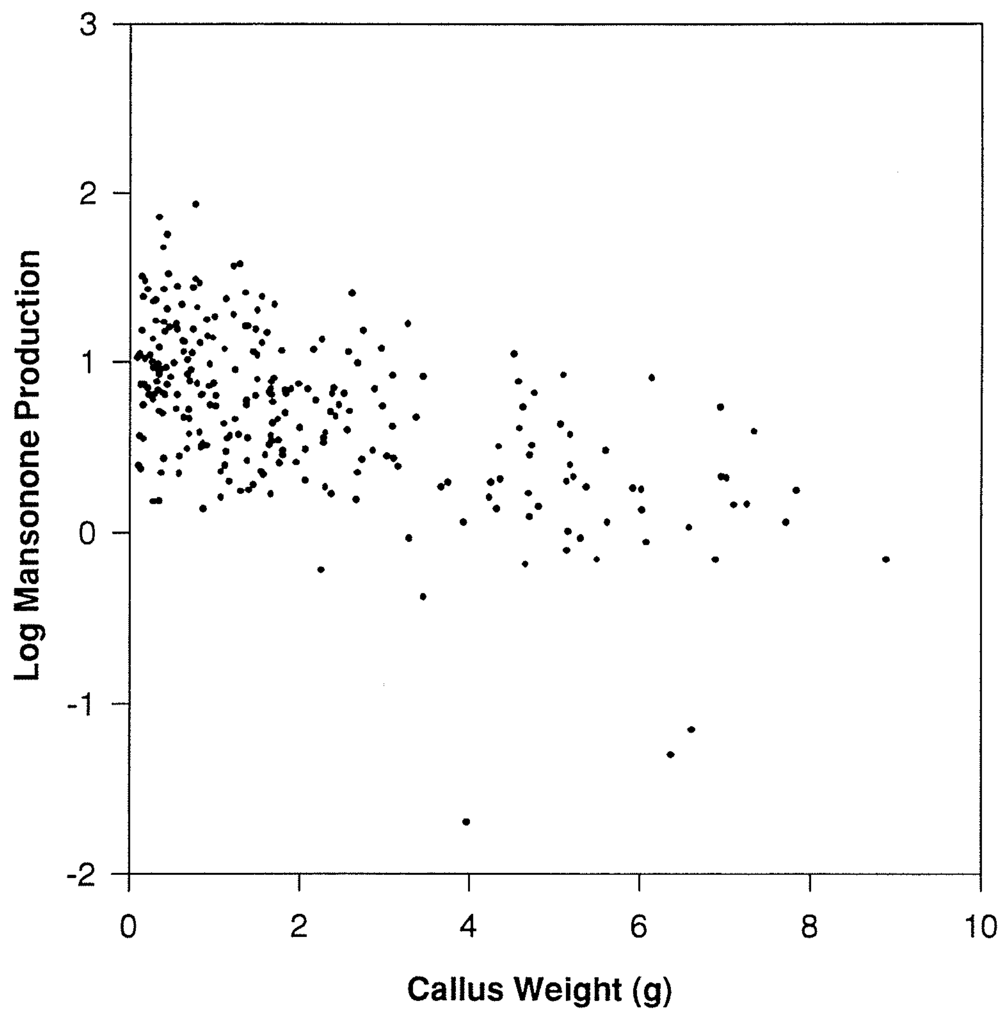


Fig. 2.8 Relationship between log mansonone production and callus weight for all samples analysed for mansonone production. $Y = -4.06 + 2.00X$. $r^2 = 0.33$.

When individual mansonones were analysed independently, it was found that the differences between parents were still significant with mansonones F and G (P = 0.0002 and P = 0.0071, respectively; Table 2.2). However, for mansonone E the difference between parents was not significant (P = 0.1457).

Mansonone levels within the different elm species and selections of American elm were generally similar to those of the wild American elm genotypes (Table 2.3). The exception was the two Japanese elms which had much lower mansonone production. When individual mansonones were analysed they seemed to be produced in similar concentrations as the wild genotypes (Table 2.4). Mansonone E was produced in much lower quantities than F and G.

When control calli were measured for mansonone production, mansonones were found to be produced in similar concentrations as in the DED inoculated pieces of calli. This result was quite surprising. The difference between inoculated and controls was that the controls had sterilized fungal growth medium added to them whereas the inoculated had *O. novo-ulmi* within the fungal growth medium. Even though the controls had produced mansonones they were omitted from the analysis because the inoculation treatments were not consistent between control and inoculated groups. Inoculated calli were used in the analysis to compare mansonone production because, independent of the elicitor, they all still had similar treatments.

Table 2.2 Mean unadjusted levels (μg/g) of individual mansonones (E, F, and G) for progeny from individual trees.

Population	Tree	N	Man. E	Std. Err.	Man. F	Std. Err.	Man. G	Std. Err.
Beausejour	1	8	0.06	0.03	6.16	2.44	2.77	1.11
	2	3	0.43	0.38	2.92	2.17	2.11	1.28
	3	4	1.41	1.06	3.41	0.60	1.77	0.85
	4	1	1.88	.	15.27	.	1.83	.
	5	12	1.27	0.39	9.99	3.17	1.24	0.27
Henderson	1	9	0.13	0.07	6.45	1.86	3.40	1.00
	2	2	0.05	0.05	0.88	0.05	1.10	0.67
	3	7	1.82	1.54	3.75	1.37	1.49	0.41
	4	11	0.13	0.03	2.39	0.36	0.89	0.09
	5	4	0.86	0.39	6.88	2.41	2.10	0.77
Lorette	1	9	1.50	1.03	8.15	2.19	3.80	1.53
	2	2	0.43	0.23	3.29	2.03	0.69	0.18
	3	2	0	0	3.54	3.54	1.13	0.40
	4	10	0.51	0.18	3.89	0.88	0.89	0.07
	5	4	0.28	0.15	4.38	0.58	2.56	0.63
Selkirk	1	6	0.48	0.13	9.53	1.43	4.85	2.13
	2	8	0.57	0.30	4.40	1.31	1.97	0.62
	3	4	1.14	0.90	2.53	1.78	4.31	2.78
St. Anne	1	6	0.50	0.33	7.85	1.37	1.88	0.64
	2	8	1.30	0.49	12.68	3.31	5.09	1.43
	3	4	2.25	1.39	1.19	0.24	1.40	0.81
	4	5	0.47	0.42	9.72	2.24	2.91	1.79
	5	2	0.08	0.07	3.77	0.95	1.63	0.77
Winnipeg	1	5	0.32	0.24	11.35	1.82	5.06	1.85
	2	7	0.04	0.04	2.93	0.88	2.56	1.12
	3	3	1.42	1.33	3.88	2.92	1.12	1.06
	4	11	0.79	0.36	4.25	0.99	0.88	0.14
	5	6	3.22	1.21	30.82	13.38	0.52	0.35

Table 2.3 Unadjusted mean mansonone levels (mansonones E, F, and G combined) for different American elm selections and elm species.

Tree	N	Mean Mansonone Production (μg/g)	Std. Err.
L' Assumption	8	5.87	4.15
Skinners	12	13.35	15.24
U. davidiana (var)	7	1.83	2.71
U. davidiana	10	1.27	0.55
U. pumila	11	4.69	5.32
U. sapporro	7	2.08	1.24

Table 2.4 Unadjusted mean levels of mansonones E, F, and G for progeny from American elm selections and elm species. N = number of progeny tested.

Tree	N	Man. E	Std. Err.	Man. F	Std. Err.	Man. G	Std. Err.
L'Assumption	19	0.51	0.21	4.15	1.33	1.21	0.18
Skinners	20	1.01	0.84	11.58	3.84	0.76	0.24
U. davidiana (var)	22	1.50	1.03	8.15	2.20	3.80	1.53
U. davidiana	16	0.12	0.01	0.66	0.12	0.60	0.08
U. pumila	24	0.42	0.35	2.66	0.96	1.62	0.40
U. sapporro	23	0.03	0.02	1.45	0.42	0.59	0.09

Inoculation of Seedlings With *O. novo-ulmi*

No external visual symptoms of DED were recorded at either site. However, at the Bird's Hill site all the parent trees within the populations had some degree of vascular staining (Tables 2.5 to 2.10). The variation between populations was significant (P = 0.0007), and the variation between parents within populations based on total staining was also significant (P = 0.0001). The range of vascular staining varied between populations. For example, staining within the Beausejour population varied from 24 to 80 % staining

while the Winnipeg population varied from 43 to 93 % (Tables 2.5 and 2.10).

Staining of the various American elm selections and elm species was generally within the range of the wild genotypes (Table 2.11). The only exception was Mord-A (American elm selection L'Assumption), which had a mean staining percentage of 95%.

Relationship Between Vascular Staining and Mansonone Production

There was no correlation between mansonone production and staining for the same parents (P = 0.60; r = 0.1). For each mansonone concentration there was a large range of percent vascular staining (Fig. 2.9). Although these comparisons do not involve the same genotypes, they do represent groups of progeny from the same parent tree.

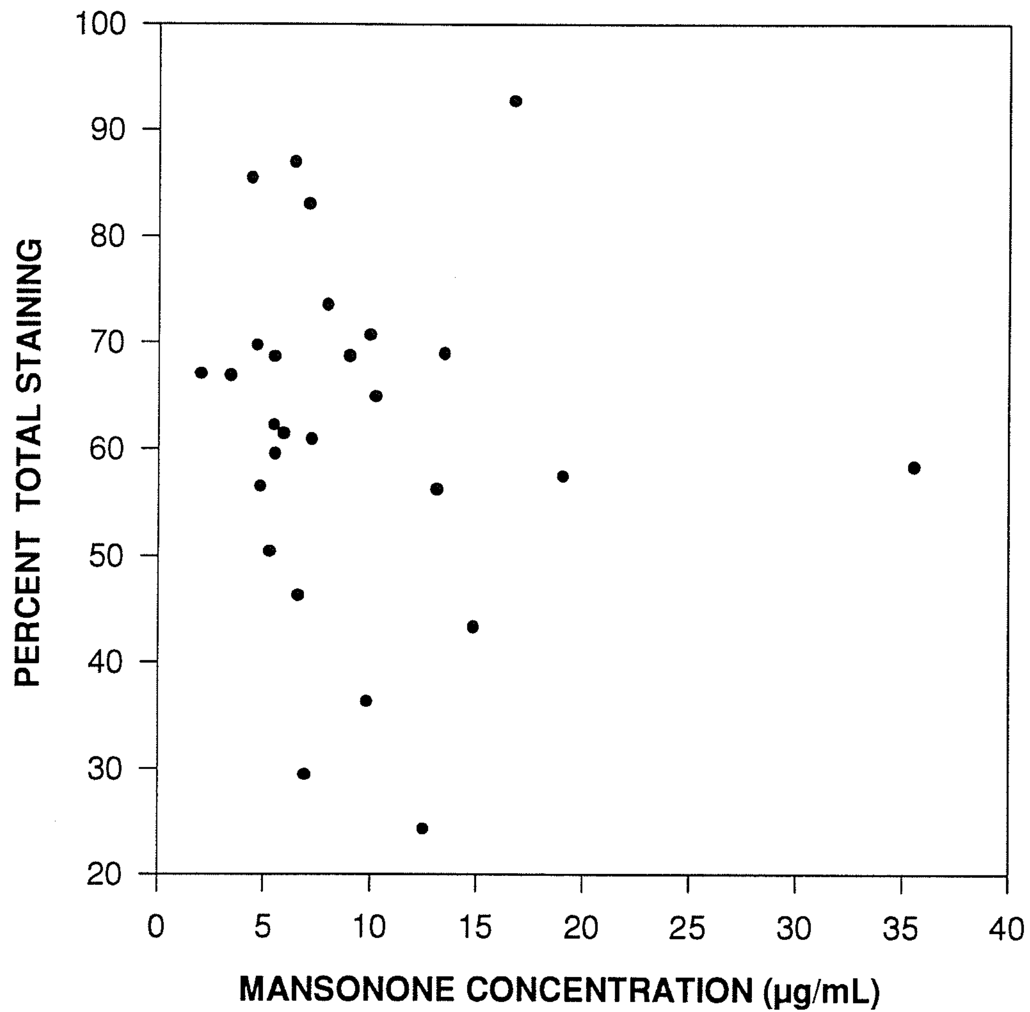


Fig. 2.9 Relationship between vascular staining and mansonone production. Data points represent progeny tested from one parent tree. However, staining data was obtained from different progeny then used for mansonone analysis.

Table 2.5 Mean percent vascular staining for progeny from trees within population Beausejour (Beau). N = number of progeny per parent tree.

Tree	N	Mean % Staining	Std. Err.
1	20	69	7.6
2	18	53	10.1
3	19	66	6.9
4	22	62	9.2
5	22	68	8.1
6	20	82	8.2
7	19	80	6.9
8	20	76	7.2
9	13	46	12.2
10	15	89	6.4
11	19	61	8.4
12	13	58	10.8
13	22	61	9.0
14	4	24	3.1
15	15	59	10.1

Table 2.6 Mean percent vascular staining for progeny from trees within population Henderson Highway (Hend). N = number of progeny per parent tree.

Tree	N	Mean % Staining	Std. Err.
1	16	89	5.7
2	21	62	8.3
3	20	71	7.3
4	17	78	8.0
5	22	77	6.5
6	20	67	9.5
7	21	78	5.4
8	21	70	8.7
9	13	83	9.6
10	20	67	8.2
11	23	50	8.7
12	22	71	7.4
13	9	40	12.9
14	9	36	13.9
15	10	53	12.9

Table 2.7 Mean percent vascular staining for progeny from trees within population Lorette (Lort). N = number of progeny per parent tree.

Tree	N	Mean % Staining	Std. Err.
1	22	73	7.6
2	19	62	8.7
3	13	69	8.5
4	20	69	8.0
5	18	85	4.9
6	22	66	8.6
7	19	70	6.8
8	24	61	7.2
9	19	65	9.8
10	20	77	6.9
11	22	63	8.9
12	15	50	10.5
13	18	61	9.6
14	21	55	8.5
15	17	61	10.3

Table 2.8 Mean percent vascular staining for progeny from trees within population Selkirk (Selk). N = number of progeny per parent tree.

Tree	N	Mean % Staining	Std. Err.
1	22	60	9.1
2	19	57	9.8
3	20	43	9.2
4	18	29	7.1
5	23	51	6.9
6	18	82	6.2
7	17	67	8.8
8	23	74	7.3
9	20	80	7.0
10	18	37	7.0
11	23	56	8.4

Table 2.9 Mean percent vascular staining for progeny from trees within population St. Anne (Stan). N = number of progeny per parent tree.

Tree	N	Mean % Staining	Std. Err.
1	21	65	8.9
2	23	65	7.1
3	21	75	7.6
4	22	58	8.4
5	18	73	7.1
6	21	45	7.7
7	14	74	9.1
8	17	56	10.7
9	19	60	8.6
10	21	56	9.0
11	18	81	6.8
12	19	47	9.4
13	22	69	8.5
14	17	69	9.0
15	19	47	9.5

Table 2.10 Mean percent vascular staining for progeny from trees within population Winnipeg (Wpg). N = number of progeny per parent tree.

Tree	N	Mean % Staining	Std. Err.
1	15	81	7.8
2	20	93	3.6
3	21	82	7.0
4	23	59	8.3
5	21	73	7.5
6	23	79	7.0
7	21	78	8.1
8	23	67	6.8
9	20	87	5.8
10	23	47	8.3
11	16	61	10.3
12	20	84	6.5
13	16	43	9.7
14	21	87	5.8
15	20	58	8.0

Table 2.11 Mean percent vascular staining for progeny from trees of American elm selections and various elm species. N = number of progeny per parent tree.

Tree	N	Total Staining Percentage	Std. Err.
L' Assumption	19	95	2.3
Skinners	20	75	7.8
U. davidiana (var)	22	53	7.8
U. davidiana	16	50	9.4
U. pumila	24	79	7.3
U. Sapporro	23	55	7.5

Discussion

Until now, there have been no studies investigating the mansonone producing ability of a large number of *U. americana* seedlings selected from the wild, derived from differing parentage. This study has shown the production of mansonones *in vitro* in *U. americana* varies significantly between genotypes. However, the main experiment in this study focused on screening a large number of genotypes, and therefore there was no replication of calli. To determine if there may be variation in mansonone production due to factors other than genotype, an experiment was performed on calli, on plates originally intended to be used in another experiment (see next chapter). Although, the design was not intended for analysis of variation within genotypes, it did provide some insight into the question. This experiment showed that differences between genotypes were greater than differences within genotypes (Appendix C), but it did not account for plate to plate variation. Nevertheless as a first step, it helps to validate that differences observed in Table 2.1 are genotypic in origin. More experimentation is needed to confirm this.

The genotypic data indicates that differences between progeny are in some cases quite large. Total mansonone production greater than 20 µg/mL was quite common and several cases of extreme mansonone production were also evident. A study by Wu *et al.* (1989) showed that mansonone levels at approximately 24 µg/mL could arrest *O. ulmi* growth by 84% *in vitro*. Several calli in this study produced mansonone amounts within that range. Other studies using callus tissue have shown strong correlations of phytoalexin levels (or concentrations) *in vitro* with those produced *in vivo* (Deaton *et al.* 1982; Helgeson *et al.* 1972). If mansonone production *in vitro* and *in vivo* is comparable,

then the levels of the mansonones produced *in vitro* in this study would be large enough to arrest growth of the DED pathogen. This problem will be addressed in future studies of *U. americana.*

The production of mansonones were significantly different among parent trees from the same population. There have been no previous reports of mansonone production differing between individual American elm trees. This result indicates that the mansonone producing ability of a tree heritable. However, it should be noted that progeny from a single tree also varied considerably.

The production of mansonones was not significantly different between populations based on the mansonone producing ability of trees within those populations. A reason why populations were not statistically different was because the large variation among trees precluded differences among populations (Fig. 2.6). Another reason why these populations were not different may be because they were chosen arbitrarily within Manitoba. There were no geographic barriers distinguishing any of the populations. This lack of isolation would not allow populations to develop independently of each other. Moreover, because of the large amount of pollen produced by elm and the relative ease by which it is transported by wind would allow for genetic exchange between the populations (Fowells 1965). The lack of geographic isolation and large amount of pollen transfer between populations may reduce genetic differences among them.

The weight of calli produced over the same time period differed among genotypes. There was a relationship between the production of mansonones and the weight of the callus. Although the relationship was variable, as the weight of calli increased the

production of mansonones per g of callus tended to decrease (Figs. 2.7 and 2.8). In other studies it was found that the ability to produce a certain weight of callus was a genetic trait (Pierik 1987). Thus the ability to produce mansonones *in vitro* could be related to the callus producing ability. Another explanation for the reduced weight of callus when mansonone production increases could be that the increased concentration of mansonones reduced the growth of the callus. Other studies have found that phytoalexins act as plant growth inhibitors as well as microorganism inhibitors (Smith 1982).

Mansonones E, F, and G were chosen because they were the three most quantitatively important mansonones. In one study, mansonones E, F and G encompassed approximately 87% of the total mansonone compliment (Duchesne *et al.* 1985). All three mansonones were found in all of the populations and parents. When the differences between populations and parents were tested with only one mansonone at a time, differences between parents were still significant for mansonones F and G. When mansonone E was tested independently, the significance between parents was not as evident as with the other two. A possible explanation for this is that mansonone E was also recorded in the smallest amounts within the total mansonone fraction. It is possible that the method used was not sensitive enough to detect this mansonone accurately. Also, since mansonone E was in the lowest concentration within the total mansonone fraction it was represented by the smallest peak. Human error in measuring such small peaks could have accounted for the non-significant differences for mansonone E.

Most of the different species and selections of elm produced mean levels of mansonone within the range of the wild genotypes of *U. americana*. *Ulmus davidiana*

var. japonica did show mean levels of mansonone which were much lower than the average. Some of the other Asiatic species were also in the low range of mean mansonone production. This lower amount of production could be due to the fact that Asiatic species of elm have a different elicitation mechanism for mansonones. Yang *et al.* (1989) noted that the accumulation of mansonones by the Asiatic elm species *U. pumila* was much slower than that of *U. americana.* Another explanation for the lower production of mansonones could be due to the fact that the Asiatic elm species are not affected by DED to the same extent. The resistance mechanism for the Asiatic species is largely influenced by their vessel morphology (Elgersma 1970). Since they can resist infection by other means, perhaps mansonone production is less important in these elms. The two *U. americana* selections produced mansonones within the general range of the other wild *U. americana.*

During this study control calli were also included in the measurement of mansonones. The assumption was that the control calli would not produce any mansonones because no fungal elicitor was added. The results from other studies have shown that a fungal elicitor must be present to elicit the accumulation of mansonones in American elm callus (Yang *et al.* 1989; Duchesne *et al.* 1994). Despite the observation that elicitation took place without the fungus, the inoculated callus samples were utilized in the experiments because all of them had similar treatments regardless of the elicitor origin. The control calli were not used as controls in this study because they produced mansonones and were not used as part of the inoculated group because they did not have the same inoculation treatment. Because of the unexpected elicitation in the control

material, non fungal elicited mansonone production became an additional subject of this thesis (Chapter 3).

Staining of elm conductive tissue when infected by the DED pathogen is a symptom also associated with the disease. The staining of the xylem tissue indicates the spread of the fungus within the tree. Several studies have used the amount of staining in an inoculated tree as an indication of the severity of the disease and resistance of the host (Sinclair *et al.* 1975; Sinclair and Larsen 1980). The resistance reaction within an elm is characterized by a compartmentalisation of the fungus to one area and subsequent halt from further movement throughout the plant. This compartmentalisation of the fungus would also halt the staining of the wood. The results of the staining experiment indicate that in this study the degree of staining varies. The total amount of staining within the seedlings varied from 0% to 100% of the stem stained. The staining was significantly different among trees and between populations (Appendix C). The significant difference in parent trees within populations was similar to the result from the mansonone experiment. However, in contrast to the mansonone experiments, populations were significantly different in their vascular staining

As outlined earlier, both vascular staining and mansonone production have been shown to be indicators of resistance. This would suggest the probability of a relationship between the two. More staining should accompany a decrease in mansonone production. Moreover, this study has shown that both of these traits differ significantly between parents. When mansonone production was plotted against vascular staining, using progeny derived from the same parents, no relationship was found (Fig. 2.9)(Appendix

C). This result indicates that at least at the juvenile stage examined, one or both of these two tests is not an accurate indicator of DED resistance. Several studies have shown that there is a juvenile resistance within young elm (Arisumi and Higgins 1961; Schreiber 1970). This juvenile resistance was the most probable cause for there being no visible symptoms of DED on the young seedlings when they were inoculated. In addition, juvenile resistance could account for the non-correlated staining and mansonone results. Young seedlings which are growing rapidly have large vessel diameters and fungal spread is unrestricted but the seedling can survive because of the rapid production of new vessels. Perhaps this could cause a weak correlation between mansonone production and staining. To address the problem of mansonone production and staining relationships, a study involving more mature trees must be used.

The American elm selections and different elm species reacted to an invasion by the *O. novo-ulmi* in a similar manner to the wild genotypes of American elm. The amount of staining was similar to that of the wild genotypes. American elm cultivar L' Assumption did show quite high amounts of staining. This could be due to the fact that it is an American elm which is susceptible to DED. The staining results for the two Japanese elm selections and *U. sapporo* cv Autumn Gold were quite low. This result corresponds with a previous study involving *U. sapporo* cv Autumn Gold in which it had low staining results compared to American elms (Green *et al.* 1985). *Ulmus pumila*, another Asiatic elm species, was higher in its percent staining but the staining in this selection was restricted to a small portion of vessels and did not spread readily to adjacent vessels. This could be due to the fact that *U. pumila* was found to have a lower amount

of vascular connections between vessels than did *U. americana* (Elgersma 1970).

When the seedlings from one parent within a population are compared to other parents within other populations there are significant differences. Such large scale differences in mansonone production have not been reported previously. There also have not been reports of how mansonone production differs among progeny from a single parent. It is known that if a trait is under genetic control, offspring of selected trees should surpass offspring of average trees (Wright 1993). This data has enhanced the idea of breeding for high mansonone producing *U. americana* genotypes. Future research in this area must concentrate on finding if there is a relationship between mansonone levels derived from *in vitro* culture and DED visible symptoms on trees mature enough to express symptoms of the disease. While studies in the past have indicated that high levels of mansonones can reduce the growth of *O. ulmi* in culture (Dumas *et al.* 1986; Wu *et al.* 1989; Proctor *et al.* 1994), the question as to whether amounts of mansonone in culture relate to resistance in the trees still remains unanswered. This question must be answered before a true breeding and selection program based on mansonones can be initiated.

Chapter 3: Mansonone Accumulation in The Absence of a Fungal Derived Elicitor in Callus Cultures of *Ulmus americana* L.

Introduction

Fungi are considered to be the main agents which elicit the accumulation of phytoalexins. Yang *et al.* (1989), using an Ontario, Canada seed source of *U. americana*, indicated that for mansonones to be produced in large amounts a fungal elicitor must be present and that abiotic elicitors were ineffective in eliciting mansonone accumulation to the same concentrations as fungal elicitors. However, it is well accepted that abiotic and other biotic elicitors can elicit phytoalexins in the same or similar concentrations as fungal elicitors (Zobel and Brown 1993; Guo *et al.* 1993; Hahn and Albersheim 1978).

In the course of a larger study of mansonone production in *U. americana* using a Manitoba seed source, mansonones were unexpectedly present in extracts of calli that had not been treated with the fungal elicitor. The calli had been treated with aseptic fungal growth medium. This led to the hypothesis that elicitation of mansonones in *U. americana* callus can occur without the addition of a fungal elicitor treatment. A series of experiments was initiated to determine the nature and timing of the non-fungal elicited mansonone accumulation.

EXPERIMENT 1.

The first experiment was designed to determine which component in the elicitation medium may have elicited the production of mansonones.

Materials and Methods

Callus Culture

Ulmus americana seed was collected in the spring of 1993 from several locations

throughout Manitoba, Canada. Callus tissue was produced as described in Chapter 1 of this thesis.

Inoculation of Callus

Two pieces of calli were inoculated with each of the 8 components of the fungal growth medium which had previously been used in the elicitation of the mansonones. One mL of each component solution, at the same concentration as in the complete medium, was placed on each piece of callus. After incubation for 24 hours, calli were frozen until mansonone extraction. Additional treatments consisting of inoculation with water only or no inoculation were used as controls. Half of these calli were frozen as usual, while the other half were analysed immediately after the 24 hour period without any freezing treatment to investigate the possibility that freezing acts as an elicitor.

Extraction and Analysis of Mansonones

Mansonones E, F, and G were extracted and analysed through High Performance Liquid Chromatography as described in Chapter 1.

Results

Mansonones were present in relatively similar quantities in all samples (Table 3.1). The presence of mansonones in both frozen and unfrozen callus tissue ruled out the possibility that mansonones were elicited by freezing. The accumulation of mansonones in the water and "no added component" treatments indicated that mansonones were

produced during the *in vitro* culture of callus and thus without the addition of an elicitor. Therefore, a subsequent experiment was initiated to determine at what stage of callus culture mansonone accumulation begins.

Table 3.1 Mean production (± standard error were applicable) of mansonone (total E, F, and G) in *U. americana* callus cultures treated with various medium components including a water control and a treatment with no added components. N= 2 samples per media component.

Medium component	Mean total mansonone production (µg/g)
$FeCl_3$	28.73 ± 27.53
$MnCl_2$	21.62 ± 8.68
Sucrose	22.56 ± 10.50
$MgSO_4$	24.36 ± 4.36
$ZnSO_4$	22.86 ± 15.58
L-Aspargine	15.07 ± 0.57
KH_2PO_4	27.84 ± 8.32
Yeast Extract	25.04 ± 0.71
H_2O	73.20
No added component	99.53

NOTE: All extracts had been frozen at -70°C prior to analysis. For completeness, a sample which had been subjected to water only and one with no added components were analysed without the freezing treatment. Their mansonone values were 47.76 and 31.54 µg/g, respectively.

EXPERIMENT 2.

Materials and Methods

Seedlings were produced and cotyledons obtained and cultured as in experiment 1. Sufficient cultures were established so that sampling could take place at specific intervals during the culture process. Cotyledons were removed from the medium at 0, 3, 24, 192, and 552 hours after cotyledon removal from the seedling and sterilization. Three plates each containing four cotyledons were used at each sampling time. The 192 and 552 hour times were chosen because they marked the physiological stages of the beginning of callus production and the browning of callus, respectively. The protocol used for extraction of mansonones and HPLC quantitative measurements was the same as experiment 1.

Results

The results of this experiment clearly showed that mansonones were produced in *in vitro* callus cultures without the application of a fungal elicitor (Table 3.1). After 24 hours there was no mansonones production. The exact time when mansonone production began was not determined but there was significant mansonone production by 192 hours and this corresponded to the beginning of callus production. The initial callus colour was green-white but as the callus tissue aged, it turned white-brown in colour. By 552 hours, the callus on all the plates had turned brown. Although variable, the accumulation of mansonones peaked and then decreased as the cultures aged (from 192 to 552 hours, Table 3.2).

Table 3.2 Mean production (± standard error where applicable) of mansonone (total of E, F, and G) in *U. americana* callus cultures with no added elicitors. N= 3 samples per sampling time.

Hours in culture	Mean total mansonone production (µg/g)
0	0
3	0
24	0
192	105.28 ± 61.19
552	72.51 ± 33.89

Discussion

In previous studies, the production of high concentrations of mansonones required a fungal elicitor (Duchesne *et al.* 1986; Duchesne *et al.* 1985; Kemp and Burden 1986). There have been no previous reports of mansonones being produced at concentrations as high as those reported here in callus tissue in the absence of a fungal elicitor. There have been reports that yeast may act as an elicitor in other phytoalexin systems (Hahn and Albersheim 1978). This led to the hypothesis that a component of the fungal growth medium, probably the yeast extract, was acting as an elicitor. However, experiment 1 revealed that the different medium components were not responsible for the elicitation of mansonones (Table 3.1). This observation is supported by a study by Zobel and Brown (1990) who demonstrated that cultured cells of carrot produced phytoalexins without the addition of a fungal elicitor. On the other hand, a previous study with similar protocol as was used in this investigation indicated that there were no mansonones produced in the

water control treatments (Duchesne *et al.* 1994; Yang *et al.* 1989).

The results of the present study indicate that the production of mansonones is correlated with the initiation of callus. It is well known that phytoalexins are wound related compounds (Harborne 1988). The fact that callus formation along the wounds of the cotyledons was first observed at the same time mansonones were first detected supports the idea that the two events are related. It is possible that the signal for wound callus production is correlated or linked with the response of plants to wounding by producing phytoalexins.

The apparent discrepancy with the findings of previous studies (e.g. Yang *et al.* 1989) is more difficult to explain. One difference between the study of Yang *et al* (1989) in which mansonones were not produced in control treatments and this present investigation is the source of the *U. americana* material. The material used by Yang *et al* (1989) came from a tree located in Toronto, Ontario. Our material came from several locations in Manitoba. The source of the material was ruled out as a possible difference in the production of non-fungal elicited mansonones because even *U. pumila* callus produced mansonones in control treatments. Yang *et al.* (1989) found that *U. pumila* controls did not produce any mansonones.

Another difference between the two studies was that Yang *et al.* (1989) had used cotyledons and hypocotyls derived from *in vitro* germinated seeds while this study used cotyledons derived from soil germinated seeds. Perhaps this difference in the source of material used for callus induction had some affect on the ability of the tissue to produce mansonones.

One of the possible factors in the elicitation of mansonones without a fungal elicitor may be related to the nature of the callus which was produced. In the present study, the callus turned brown in all replicates by 552 hours in culture. The browning of callus tissue has been associated with the accumulation of phytoalexins in several other plants (Latunde-Dada and Lucas 1985; Zobel and Brown 1993). Although the mansonones were produced before browning, the tendency to turn brown may be important because the cultures of Yang *et al.* (1989) remained white throughout their experiments (M. Hubbes, Pers. Comm.). Latunde-Dada and Lucas (1985) found that more phytoalexins accumulated in resistant alfalfa callus lines than susceptible lines and that resistant callus turned brown when treated with a fungus as a result of phytoalexin accumulation. Therefore, when the callus in this experiment turned brown it may have resulted from phytoalexin accumulation from some abiotic elicitor.

The browning of callus during culture has been shown in some cases to indicate non ideal culture conditions (Pierik 1987). Perhaps factors such as agar, nutrient or hormone source and concentration as well as tissue handling could cause the callus to brown and thus accumulate mansonones.

The trend for a decrease in mean mansonone production as the cultures aged was also reported by Yang *et al* (1989). As the length of subculture interval increased mansonone production decreased (Yang *et al.* 1989). A possible explanation for this is that the production of mansonones has peaked at some earlier time and the mansonones are leached out of the callus into the culture medium as the culture ages.

This study indicates that testing for mansonones *in vitro* must be undertaken

carefully, and under the correct environmental conditions, to obtain the highest degree of accuracy. Haberlach *et al* (1978) showed that the disease resistance of tobacco callus can be modified by changing the balance of auxins and cytokinins in the culture medium. Therefore, the ability to optimize the use of callus cultures *in vitro* depends on the ability of the researcher to grow it on a medium in a proper environment so that elicitation does not take place until it is inoculated with the desired pathogen. This problem is being addressed in a follow-up study.

SUMMARY AND CONCLUSIONS

High concentrations of mansonones *in vitro* cause a reduction in growth of the DED fungus *Ophiostoma ulmi* (Wu *et al.* 1989). These toxic effects of mansonones have led some researchers to conclude that they are associated with the resistance mechanism of the tree (Duchesne 1993; Hubbes 1993). There have been no previous studies which have looked at the production of mansonones over a large group of related seedlings. In this study six populations within Manitoba were chosen and seedlings were collected from several trees within each population. This study provides evidence that the production of mansonones within seedlings derived from the same parent tree differed significantly from those derived from another tree from the same population. This is the first report that mansonone production ability is translated from parent trees to their seedlings. Moreover, this study has also shown that genotypic variation in mansonone production is quite large in some instances and that genotypic differences are in fact real differences. No significant differences were found between the populations.

Seedlings from the same parent trees which were tested for mansonone production were tested for symptoms of DED following inoculations. Vascular staining differed significantly in progeny from one parent to those of another parent within the same population. This result was similar to the one derived for mansonone production. Although when a correlation test was performed to investigate if mansonone production and staining were related, no relationship was found. Since both of these have been

suggested to be indicators of resistance or tolerance to DED, one or both of them is not accurate when the tree is in a juvenile stage. Future research in this area must concentrate on mature trees. Mansonone production should be measured in trees which are older than three years followed by inoculation with DED and analysis of both visual symptoms and vascular staining. Trees older than three years reportedly lose their juvenile resistance and thus show visual symptoms of DED (Townsend and Santamour 1993). Such research is necessary to determine if there is a true relationship between mansonone production *in vitro* and DED tolerance in elm.

The largest confounding result in this study was the non-fungal elicited production of mansonones within the control calli. Subsequent experiments performed to address this problem concurred that the production of these mansonones was not as a result of the sterilized fungal growth medium used for controls or the fact that the production of callus through the use of cotyledons caused the elicitation. The technique for callus production was generally similar to that of previous studies (Yang *et al.* 1989). Prior to further research into *in vitro* mansonone production and its possible relationship to tolerance to DED, the problem of the non-fungal elicited mansonone production within callus cultures must be solved. Future work should concentrate on four areas: material used for callus production, the medium components, technique of culturing and the growth environment. The material used for culture should be investigated because this study is the only one which has used elm material from such a vast number of parents. Perhaps the elm material used in this study was different in its sensitivity to growth medium components. Callus growth medium components must be checked to see if they are responsible for the

production of mansonones. Perhaps one of the components of the media used for culture in our lab has been contaminated and is causing elicitation. The technique of collecting material for culture must also be investigated. In previous studies cotyledons and hypocotyls from *in vitro* germinated seed was used for callus production while in this study cotyledons removed non-destructively from young seedlings grown in the greenhouse were used. Finally, the growth environment will be studied to see if it had or has an affect on the production of mansonones within callus.

The large range of mansonone production between genotypes provides hope that a high mansonone producing genotype can be selected. The only problem is that this mansonone production was measured *in vitro* and not *in vivo*. Additional research must concentrate on finding a relationship between mansonone production *in vitro* and its effects on the tolerance of the tree to DED. This research should also include testing for mansonone production within infected branches to give an idea of the production of mansonones *in vivo*.

A relationship was also found between the ability to produce mansonones and the weight of callus produced within a genotype. As the weight of the callus went up the ability to produce mansonones decreased. Experiments were performed to determine if the low production was a result of lack of elicitation in the centre of the piece of callus but no difference was found (data presented in Appendix E). Future research in this area should continue to see if there is a link between the ability to produce mansonones and the ability to produce callus. A link could be possible because both of these factors are a result of a wound reaction.

The ability to measure mansonones quickly and accurately through the use of HPLC has increased the speed of research in this area. The improved protocol developed in this study was based on the preliminary work developed in another laboratory (Dumas, Pers. Comm.). The new protocol is quicker and utilizes a much more simple HPLC system which is readily available. This HPLC analysis of mansonones has enabled this study to measure a large number of samples which would no be possible using TLC. The new HPLC protocol in conjunction with an autosampler would allow a large number of samples to be analysed and thus facilitate mansonone research even more.

Throughout this study different elm species and various selections were included in the experiments. Generally the species and selections acted in the same manner as the *Ulmus americana* selected from the wild. Exceptions to this were the two *Ulmus davidiana* var. japonica selections and *Ulmus sapporro* which all had low amounts of staining and also produced the least amount of mansonones. It is also known that the Asiatic elms have a greater amount of resistance than does the American elm. Perhaps the reason why low amounts of mansonone are associated with resistant species is because in these elms there is a different mechanism of resistance than in the American elm. Future work with these species would also involve mature trees to answer the question are mansonones involved in Asiatic elm resistance.

This study has provided a framework for mansonone research in the future. It has contributed vital information with regards to variation in mansonone production between genotypes. Before mansonones can be used as an indicator for tolerance to DED within *Ulmus americana* many questions must be answered. The first question to be addressed

is the one of non-fungal elicited mansonone production within callus cultures of *Ulmus americana*. With continued research in this field perhaps a definitive answer can be found to the question of mansonone involvement in DED tolerance.

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APPENDICES

APPENDIX A: B_5 Plant medium used for callus growth of *Ulmus americana*.

Macro-nutrients (mg L^{-1})		**Micro-nutrients (mg L^{-1})**	
KNO_3	2500	KI	0.75
$MgSO_4.7H_2O$	250	$MnSO_4.H_2O$	10
$NaH_2PO_4.H_2O$	150	$NaMoO_4.2H_2O$	0.25
$CaCl_2.2H_2O$	150	$CoCl_2.6H_2O$	0.025
$(NH_4)_2SO_4$	134	H_3BO_3	3.0
Hormones (mg L^{-1})		$ZnSO_4.7H_2O$	2.0
2,4-D	1.0	$CuSO_4.5H_2O$	0.025
Kinetin	1.0	Fe EDTA	0.043
Miscellaneous (mg L^{-1})		**Organics (mg L^{-1})**	
Potato Dextrose Agar	20,750	Nicotinic Acid	1.0
Agar	4,500	Pyridoxine HCl	1.0
Distilled H_2O	to 1 L	Thiamine HCl	10
		Inositol	100
		Sucrose	20,000
		Cocomilk	50

APPENDIX B: Takai and Richard's medium used for *Ophiostoma ulmi* culture.

Components (mg L^{-1})		**Components (mg L^{-1})**	
KH_2PO_4	1,000	$MgSO_4.7H_2O$	100
$FeCl_3.6H_2O$	0.48	$ZnSO_4.7H_2O$	0.44
$MnCl_2.4H_2O$	0.36	L-asperagine	2,000
Sucrose	10,000	Yeast Extract	2,000
Distilled H_2O	to 1 L		

APPENDIX C: Analysis of variance (ANOVA) tables.

Table C-1. ANOVA for total mansonone production within wild genotype parent trees and populations.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Population	5	840.06	168.01	1.74	0.1295
Parent within population	22	5837.03	265.32	2.75	0.0002

Table C-2. ANOVA for total mansonone production within wild genotype parent trees and populations using weight of callus as a covariate.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Population	5	818.73	163.75	1.82	0.1140
Parent within population	22	5000.69	227.30	2.52	0.0006
Weight of callus	1	941.55	941.55	10.44	0.0016

Table C-3. ANOVA for mansonone F production within wild genotype parent trees and populations.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Population	5	691.29	138.26	2.10	0.0691
Parent within population	22	3993.40	181.52	2.76	0.0002
Weight of callus	1	339.89	339.89	5.17	0.0246

Table C-4. ANOVA for mansonone E production within wild genotype parent trees and populations.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Population	5	6.22	1.24	0.49	0.7837
Parent within population	22	76.11	3.46	1.36	0.1457
Weight of callus	1	3.53	3.53	1.39	0.2408

Table C-5. ANOVA for mansonone G production within wild genotype parent trees and populations.

Source of variation	Degrees of freedom	Sums of square	Mean square	F value	Pr > F
Population	5	36.29	7.26	1.16	0.3305
Parent within population	22	280.54	12.75	2.04	0.0071
Weight of callus	1	107.53	107.53	17.24	0.0001

Table C-6. ANOVA for percent total vascular staining within wild genotype parents and populations.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Population	5	2.79	0.56	4.26	0.0007
Parent within population	80	24.99	0.31	2.39	0.0001

Table C-7. ANOVA for total mansonone production between genotypes.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Genotype	11	2910.05	264.55	24.47	0.0001

Table C-8. ANOVA for total mansonone production differences in control v.s. inoculated comparison.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Treatment	194	21658.94	111.64	0.27	0.6017

Table C-9. ANOVA for total percent staining differences among different American elm selections and elm species.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F value	Pr > F
Parent trees	5	3.09	0.62	5.54	0.0001

APPENDIX D: The effect of cotyledon removal from seedlings of *Ulmus americana* on total height of the seedling.

Introduction

As part of a larger study cotyledons were to be removed from seedlings of *Ulmus americana* for the production of callus tissue. This method of callus production was chosen as a non-destructive means of producing callus from a seedling. The objective of this study was to investigate the effect of early removal of the cotyledons had on eventual height growth of the seedling.

Materials and Methods

Ulmus americana seeds were planted in a flat containing Metro Mix ™. Both cotyledons were removed from four seedlings at 4, 8, 12, 16, 20, 24, 28, and 32 days after germination. A control group was also included in the study in which the cotyledons were not removed. The seedlings total height was measured 42 days after germination.

Results

After 42 days of growth the cotyledons on the control seedlings had turned yellow and were beginning to senesce. All of the seedlings survived the removal of cotyledons and all seedlings had total heights similar to control seedlings (Table D-1).

Table D-1. Effects of cotyledon removal on *Ulmus americana* seedling height.

Cotyledon Removal Treatment	Seedling 1 Height (mm)	Seedling 2 Height (mm)	Seedling 3 Height (mm)	Seedling 4 Height (mm)
Control	90	88	69	84
Day 4	82	89	77	93
Day 8	73	102	80	114
Day 12	90	111	118	111
Day 16	86	106	99	168
Day 20	186	109	158	110
Day 24	164	90	72	79
Day 28	126	108	109	73
Day 32	128	110	107	101

Discussion

Removal of cotyledons from *Ulmus americana* seedlings at various times after germination did not affect the total height of the seedlings in this study. All of the seedlings measured were within the same height range and were often greater than the control. In another study where cotyledons were removed 8 days after germination, it was found that total height did decrease (Marshall and Kozlowski 1974). Nevertheless, seedlings in that study did not die and it was also noted that compared to other species the photosynthetic role of *Ulmus americana* cotyledons seemed to be minor (Marshall and Kozlowski 1974). These data, along with the information gathered from this experiment, indicates that the removal of cotyledons from seedlings of *Ulmus americana* for use in tissue culture would not affect the growth of the seedling significantly. Therefore, for the

experiments outlined in Chapters 1, 2 and 3, cotyledons were removed 7-10 days after germination. At this point the seedlings would survive and the young healthy leaf tissue would produce callus rapidly.

APPENDIX E: The effect of callus size on the mansonone production ability of large pieces of calli.

When mansonone production was found to be lower in larger pieces of calli (chapter 2), one hypothesis was that cells towards the centre were not receiving any elicitor due to the large size. An experiment was designed in which three large pieces of calli were inoculated with the pathogen and then divided into outside and inside portions. Each portion was analysed for mansonone production. There was no difference in the production of mansonones between the inside and outside portions (Table E-1). Therefore, it is probable to conclude that all cells within the calli received an elicitation signal. Chapter 3 has illustrated that mansonone accumulation within callus does not require a fungal elicitor, but regardless of the elicitor origin the cells within the centre of the calli are receiving an elicitation signal.

Table E-1. Comparison of mansonone production from the inside and outside portion of a piece of callus.

Genotype	Inside Mansonone Production (μg/g)	Outside Mansonone Production (μg/g)
Hend-10 #7	2.58	2.51
Stan-5 #5	1.93	2.30
Wpg-12 #13	3.16	3.32