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Induced resistance for the control of Dutch elm disease

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Abstract

In 1980, our research group at the faculty of Forestry, University of Toronto, demonstrated that young elm seedlings (4 years old) acquired resistance against aggressive strains of the Dutch elm disease pathogen (*Ophiostoma novo- ulmi)* when they were first inoculated with non-aggressive strains (*O. ulmi)* of the pathogen. This work was repeated in 2000 and 2002 with elm trees between 15 and 20 years of age. These trees were stem inoculated at breast height with *O. ulmi* and challenged with *O. novo- ulmi*, imitating the infection process by the European elm bark beetle. In general, the experiments indicated that non-aggressive strains, inoculated into the stem of 15-20-year-old conditioned trees, may produce disease symptoms but trees generally survive. This is in contrast to the unconditioned controls. These experiments further indicate that this type of induced resistance does not confer absolute immunity or protection under all circumstances but reduces considerably the disease severity and protects the tree from super-infection. Work based on induced resistance in elms in our laboratory led to the isolation of a glycoprotein that elicited defense mechanisms in the tree. Determination of the amino acid and DNA sequence of the glycoprotein resulted in the characterization of the elicitor gene. Field experiments on 5-year-old elm seedlings and 10 to 15 year old trees led to the following conclusions: the success of the elicitor treatment in protecting the tree against pathogen attack depends on the genetic constitution of the tree, its health and on environmental conditions.

Key words: virulence, elicitors, biological control, *Ulmus americana, Ophiostoma ulmi.*

Resumen

Resistencia inducida para el control de la grafiosis del olmo

En 1980, nuestro grupo de investigación en la Facultad de Ciencias Forestales de la Universidad de Toronto demostró que brinzales jóvenes de olmo de cuatro años de edad adquirieron resistencia frente a la cepa agresiva del patógeno de la grafiosis (*Ophiostoma novo-ulmi)* cuando previamente se habían inoculado con la cepa no agresiva (*O. ulmi)* del patógeno. Este trabajo se repitió en 2000 y 2002 con olmos de entre 15 y 20 años de edad. En ellos se inoculó en el tronco, a la altura del pecho, con *O. ulmi* y posteriormente *O. novo-ulmi*, imitando el proceso infectivo realizado por los barrenillos del olmo. En general, el ensayo mostró que las cepas no agresivas, inoculadas en el tronco de árboles de 15 a 20 años, pueden producir síntomas de la enfermedad, pero que los árboles por lo general sobreviven, en contraste con los controles. Estos ensayos indican además que este tipo de resistencia inducida no confiere inmunidad absoluta o protección en todas las situaciones, pero sí que reduce considerablemente la severidad de la enfermedad y protege al árbol de infecciones más grave. El trabajo basado en la resistencia inducida en los olmos en nuestro laboratorio condujo al aislamiento de una glicoproteína que induce mecanismos de defensa en los árboles. La determinación de los aminoácidos y de la secuencia de ADN de la glicoproteína permitió la caracterización del gen inductor. Ensayos de campo en brinzales de olmo de cinco años y en árboles de 10 a 15 años condujeron a la conclusión de que el éxito del tratamiento inductor en la protección del árbol frente al ataque del patógeno depende de la constitución genética del árbol, de su salud y de las condiciones ambientales.

Palabras clave: virulencia, control biológico, *Ulmus americana, Ophiostoma ulmi.*

Introduction

The concept of acquired physiological immunity in plants is not new. In fact, in 1901, Ray and Beauvery, two French scientists, were among the first to suggest that plants display immunological reactions comparable to those in animals. But it was not until 1933 when Chester critically reviewed the literature on the phenomenon of acquired immunity that this concept gained greater attention and credibility. Today this view has obtained further support. Application of new tools

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of molecular biology revealed similarities between innate pathogen defense systems of plants, animals and insects (Brunner *et al*., 2002; Conrath *et al*., 2002; Nürnberger and Brunner, 2002).

Formerly, there was great skepticism about the concept of acquired immunity in plants. Therefore, Fischer and Gäumann (1929) limited the term immunity to the type of reaction found in animals. They considered immunity as the ability of the host to withstand infection as a result of a stimulus either caused by the parasite or by the introduction of protective substances. In 1950, Gäumann distinguishes between acquired immunity and induced immunity. In acquired immunity, recovery from infection protects against re-infection whereas in induced immunity an existing infection protects against super-infection. Today, the latter phenomenon is known as induced resistance which can be expressed locally in tissues surrounding the point of treatment or expressed in tissue distantly from the point of treatment as in systemically acquired resistant (SAR) (Ryals *et al*., 1994; Heath, 1995; Dong, 1998; Waterhouse *et al*., 2001).

Higher resistance in a given host plant can be induced either by inoculation with a low virulent strain of a pathogen or by treatment with specific natural or synthetic compounds termed as elicitors. All plants possess the genetic information for disease defense; therefore, susceptibility is the exception rather than the rule (Heath, 2000). Furthermore, this genetic information activated by elicitors is translated into a complex signal transduction pathway that mediates the formation of specific defense compounds produced by resistant as well as by plants in which resistance has been induced. Generally, many of these compounds are family, genera or species specific, ranging from low molecular weight compounds, specific proteins, enzymes, glycoproteins and phenolic polymers, just to name a few. Not all defense compounds have to be highly active against all pathogens to provide resistance, but when synthesized by the host in the proper sequential manner, they form a very effective multi-barrier defense system (Kuć, 1995). In addition, the efficiency of induced resistance depends also on the quality and duration of the inducing signal and on the capacity of the plant to switch quickly from its normal metabolism to biochemical processes producing effective defense barriers. These events are not only influenced by genetic factors but also by environmental conditions. Therefore, induced resistance is a correlated yet variable interplay between the triggering agents, the host's genetics, its physiological health status and the environment (Hubbes, 2001).

The molecular mechanisms of induced resistance, particularly in trees, are far from being well understood. We can only speculate that they are, at least on a cellular level, similar to some mechanisms detected in annual plants. However, too many facts are here also unknown (Conrath *et al*., 2002; Nürnberger and Brunner, 2002; Heil and Baldwin, 2002). Nevertheless, induced resistance against Dutch elm disease (DED) is a very attractive form of potential disease control. It is environmentally friendly, poses a low risk to human health, can be economical, and provides the possibility to protect elm trees against DED. This type of control would bring relief to the existing elm populations planted as shade trees until they could be replaced by disease tolerant elms.

The hypothesis to protect elms against DED by induced resistance

Numerous attempts to control the disease have concentrated on three areas: reducing the vector populations, namely the elm bark beetles (Lanier, 1978; O'Callahan and Fairhurst, 1983; Jin *et al*., 1996; Webber, 2000), extensive application of fungicides (Stipes, 2000; Stennes, 2000), and the exploitation of natural host resistance (Ouellet and Pomerleau, 1965; Holms, 1976; Heybroek, 1993; Smalley and Guries, 1993; Smalley *et al*., 1993; Townsend, 2000).

Control of elm bark beetles via chemical insecticides still seems the preferred choice in areas of high beetle populations to reduce the inoculum potential. However, in the long run this option is not viable because of its negative impact on the environment. Up to now, vector control did not gain the expected success (Sticklen *et al*., 1991). Other, biological solutions are therefore investigated (Heybroek, 2000; Webber, 2000).

The introduction of several benzimidazole systemic fungicides has prompted a number of investigations on the efficacy of these compounds for DED control. Stipes (2000) and Stennes (2000) report success and difficulties experienced with fungicide treatments. Alamo and Arbotect-S applied as therapeutic treatments can be effective when used properly, but economic reasons are hindering large-scale treatments.

The initial notion was that developing elm trees with genetic resistance to DED is a lengthy and uncertain

process (Ouellet and Pomerleau, 1965; Holmes, 1976). Heybroek (1993), Smalley and Guries (1993), Smalley *et al*. (1993), Ware and Miller (1997) and Smalley and Guries (2000) produced results that were more optimistic. Lately a number of elm selections have been released that appear very promising (Townsend, 2000). The problem with these selections is the lack of understanding of the genetic mechanisms that render them resistant against the DED fungus, and therefore, no estimates can be made as to whether this resistance will last or not. Small changes in the genetic background of the fungal populations or in the physiology of the host as it ages may cause a loss of resistance. Some of the members of the, initially resistant «Liberty» elms, are now being attacked by the fungus. (A.L. Shigo, personal communication).

Field observations show that some trees have the means to defend themselves successfully against the invasion of the DED pathogen by restricting the spread of the fungus in their vessels as seen on p. 13, Fig. 1 in «Dutch Elm Disease-The Early Papers» by Holms and Heybroek (1990).This is one of the first photographs of DED in Holland. It shows a stem disc infected by the DED fungus. The tree concealed the fungus successfully in the vessels of at least four annual rings. A similar defense reaction is shown in Figs. 37 and 69 in «Compendium of Elm Diseases» by Stipes and Campana (1981).These Figs. depict the successful defense reaction of the tree by confining the invader in its xylem. We assumed that if the mechanisms of these defense reactions could be clarified and the genetic basis understood they might well form a solid basis for disease control and breeding for resistance (Hubbes, 1981, 1999).Therefore, our efforts in Toronto concentrated on the expression of defense mechanisms of elms in response to fungal infection.

In 1981, we postulated that the defense mechanisms of elms can be increased to become tolerant to the DED pathogen. This hypothesis was based on the

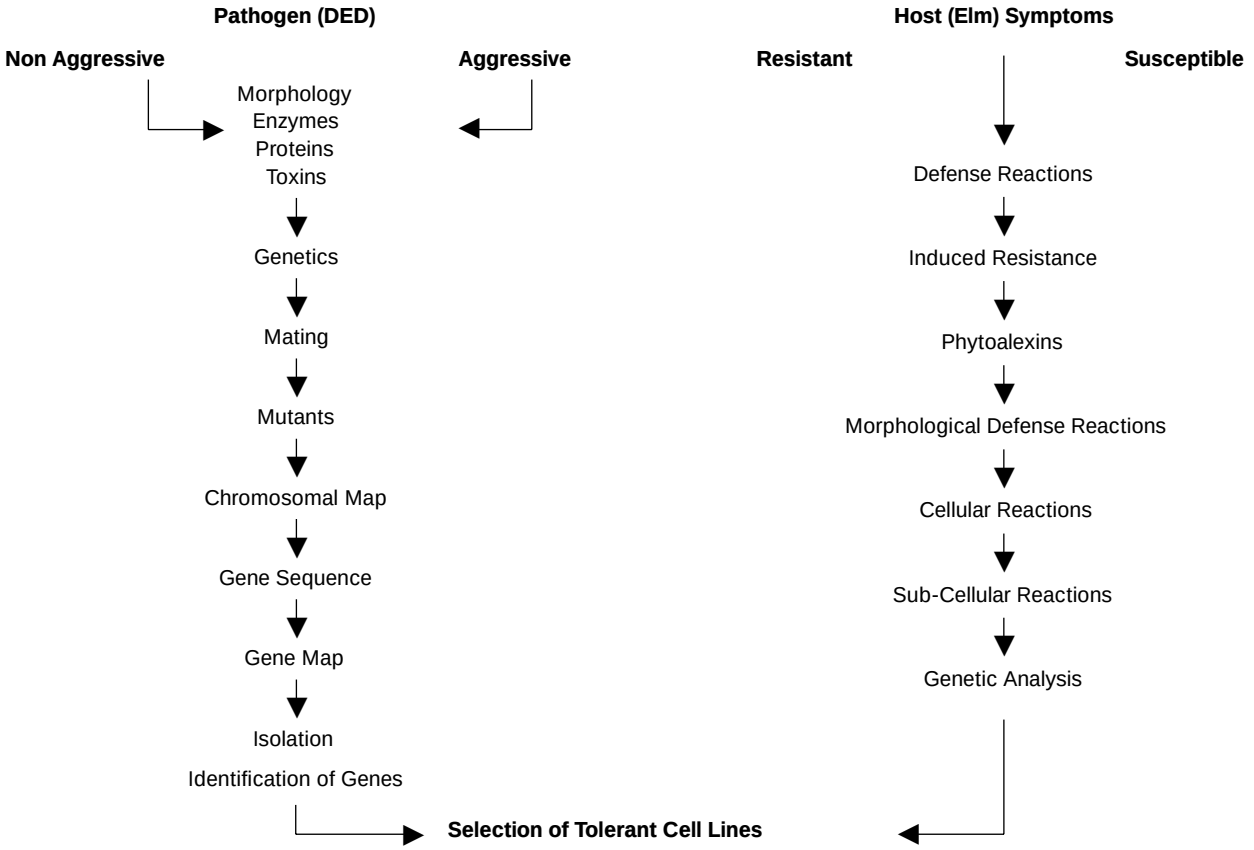

Figure 1. Hypothesis proposed in 1981 for the biological control of Dutch elm disease (DED). Defense mechanisms of elms can be enhanced to coexist with compatible strains of the DED pathogen. Thus, objectives are to produce low virulent pathogen strains, and to produce elms with strong defense mechanisms.

discovery that young elm seedlings (4 years old) acquired resistance against high virulent strains when first inoculated with a low virulent (non-aggressive) strain of the pathogen (Hubbes and Jeng, 1981). Scheffer *et al*. (1980) had also reported similar results. We formulated a new strategy for the control of DED that contrasts the traditional chemical method (Fig. 1). It proposes the inoculation of elms with a low virulent strain of the pathogen. We suggested the rearing of elm bark beetles under controlled conditions on elm logs infected with *O. ulmi*. The emerging beetles, contaminated with fungal spores, by feeding on healthy trees would introduce into these the non-aggressive strain and trigger their defense system against subsequent ingress of the aggressive strains. However, when we suggested this to the City Forester of the City of Winnipeg, a lot of skepticism was manifested concerning this type of control strategy. Many facts in the biology and in the life cycle of the pathogen were not known. A major concern was that the non- aggressive strain (*Ophiostoma ulmi*) might cross with aggressive strains (*O. novo-ulmi*) and produce more virulent offsprings. However, experimental data published by Brasier and Gibbs (1976) showed that no increased virulence occurred following strainal crossing. At that time, our knowledge on the pathogens' mating systems and factors that control their virulence was sparse.

Therefore, our long- term objectives were: 1) to produce low, virulent pathogen strains and 2) elms with strong defense mechanisms. Our group began this work with the molecular characterization of *O. novo-ulmi* (aggressive) and *O. ulmi* (non-aggressive) strains. Simultaneously comparative studies between resistant and susceptible elm hosts were initiated. Results of these investigations have been reviewed by Bernier (1993), Duchesne (1993), Jeng (1993), and Hubbes (1993; 1999). Experiments were carried out to identify pathogen factors that trigger measurable cellular and host tissue responses. Inoculation experiments revealed that elms responded to the invading pathogen by producing of specific fungi toxic compounds, which were identified as mansonones. The implication of these compounds in the defense reactions created as much discussion as for the implication of cerato-ulmin in fungal virulence (Guries and Smalley, 2000). The fact is that these two metabolites are inevitably correlated with the host pathogen interactions in the DED complex, although their exact importance is still to be discovered Hubbes (1999).

Elicitors and induced resistance in American elms

Elm seedlings respond to pathogen attack by producing mansonones as already reported (Dumas *et al*., 1983; Jeng *et al*., 1983).We developed a bioassay to measure this host response further and in view of isolating fungal components that may trigger defense reactions similar to those encountered in the elm host under natural conditions. The production of mansonones seemed a reliable and measurable parameter to test strainal virulence as well as fungal fractions that induce this reaction (Yang *et al*., 1989; Hubbes, 1999). Based on this bioassay, components were isolated from the cell wall, the cytoplasm and the culture filtrate that elicited the production of mansonones. The primary objective was to characterize differences in the virulence of fungal isolates. A spin off from these experiments might ensue as relevant concerning the use of a living low virulent pathogen strain for DED control. We speculated that if the isolated elicitor could have a similar effect in inducing resistance in the elm host as the fungus does then there would be no further concerns.

First, the fungal fraction that we planned to utilize for the experiments of induced resistance had to be precisely characterized. For this purpose, the culture filtrate fraction that had already been relatively well purified (Yang, 1991), and determined was selected. This glycoprotein was purified by additional polyacrylamid gel electrophoresis to the point that an amino acid sequence was obtained. Primers were designed from the amino acid sequence. Genomic DNA was extracted from lyophilized budding cells and used in PCR reactions with the primers. The PCR product from each reaction was cloned into a TA cloning vector. DNA sequencing was performed using double-stranded PCR-derived DNA which was sub-cloned into a plasmid vector. Sequence reactions were carried out with a T7 Sequencing kit (Pharmacia) using *S 35 dATP and electrophoresized in a model S2 sequencing gel electrophoresis apparatus. The universal and reverse primers were used as sequencing primers to determine the DNA sequence of the elicitor (Hubbes 2000, United States Patent 6,160,100). According to the amino acid sequence and the DNA structure, the elicitor falls into the group of aspartic proteinases. Its amino acid sequence and nucleotide sequence is given below.

Amino acid Sequence: 433

MAPLTHFLAAATLAGLASAVPTARDNVKVGSTTLHQVRNTNYTFNGAVSV
50
YKTYLKFGAAIPEQLQAAVDNTGLLSKRTSGSAVATPIDSSDDAYSIPVS
100
IGTPAQVLNLDLDTGSSDLWVFSSLTPSSEVNGQSVYTPTKSTTSKLVSG
150
ATWQVSYGDGSSSSGVIYTDKVTIGGITAASQAVEAAKVVSSSFTSDSSI
200
DGLVGLGFDSLNSASPSAVPTFFDNIIGSLDKPVFTADLKHNKAGSYDFG
250
VIDSSKYTGALTYVPVNTDPGYWTFTSSGYGIGTAAFKSTSVTGIADTGT
300
TLLYLDTAIVKAYYAQISGSSNSATTVATFSSALPPPLIYFGVGSARITI
350
PGSYINYGPVTPAAPLASAVCRTARILASTSLAMLPLRLLSLFSMVPAAP
400
VWVGHPRPCKQFTVLQSPTSQAIPARQKTRMF

Total length = 433

CDNA nucleotide sequence: 1,309 base pairs

ATGGCT	CCTCTCACCC	ACTTCCTCGC	CGCTGCCACC	CTGGCCGGGC	TCGCCTCCGC
TGTGCCCACT	GCCCGTGACA	ACGTCAAGGT	CGGCAGCACG	ACCCTTCACC	AGGTCCGCAA
CACCAACTAC	ACCTTTAACG	GTGCTGTCTC	AGTCTACAAG	ACCTACCTCA	AGTTTGGCGC
TGCCATTCCC	GAGCAACTAC	AGGCTGCCGT	CGACAACACT	GGTCTCCTGT	CCAAGCGCAC
CAGTGGAAGT	GCCGTCGCCA	CTCCCATTGA	CAGCTCCGAC	GATGCCTACT	CCATCCCTGT
CAGCATTGGT	ACCCCTGCCC	AGGTTCTGAA	CTTGGACTTG	GACACTGGCT	CGTCTGATCT
ATGGGTCTTC	AGCAGCCTTA	CTCCTTCGTC	TGAGGTCAAT	GGCCAATCGG	TCTACACTCC
TACGAAGAGC	ACCACCTCCA	AGCTAGTCTC	TGGCGCCACC	TGGCAGGTCT	CCTATGGCGA
TGGCTCGTCG	TCCAGTGGTG	TCATCTACAC	TGACAAGGTC	ACCATTGGCG	GCATCACTGC
TGCCAGCCAG	GCTGTTGAGG	CTGCCAAGGT	TGTTTCTTCT	TCCTTCACCT	CCGACAGCTC
CATCGATGGC	CTCGTCGGTC	TGGGCTTCGA	CAGCCTCAAC	TCCGCCTCCC	CCAGCGCTGT
GCCCACTTTC	TTCGACAACA	TCATTGGTAG	CCTGGACAAG	CCCGTTTTCA	CTGCTGATTT
GAAGCACAAC	AAGGCCGGTT	CATACGACTT	CGGTGTTATC	GACAGCTCCA	AGTACACCGG
CGCCCTGACC	TACGTTCCTG	TTAACACCGA	CCCCGGTTAC	TGGACATTCA	CCTCGTCTGG
CTACGGAATT	GGAACTGCTG	CTTTCAAGTC	CACTAGCGTC	ACTGGTATTG	CCGATACCGG
TACTACCCTG	CTGTACCTCG	ACACCGCCAT	CGTCAAGGCC	TACTACGCAC	AGATCAGCGG
TTCGTCCAAC	AGCGCTACTA	CGGTGGCTAC	GTTTTCAAGT	GCTCTGCCAC	CCCCCCTGAT
TTACTTCGGT	GTCGGCAGTG	CCAGAATTAC	TATCCCCGGT	AGCTACATTA	ACTACGGCCC
CGTCACTCCG	GCAGCACCAC	TTGCTTCGGC	GGTCTGCAGG	ACAGCTCGGA	TATTGGCATC
AACATCTTTG	GCGATGTTGC	CCTTAAGGCT	GCTTTCGTTG	TTTTCGATGG	TTCCAGCAGC
CCCCGTCTGG	GTTGGGCATC	CAAGACCCTG	TAAGCAGTTC	ACTGTATTGC	AATCGCCAAC
AAGCCAAGCC	ATCCCAGCTC	GACAAAAAAC	AAGGATGTTT	TGA	

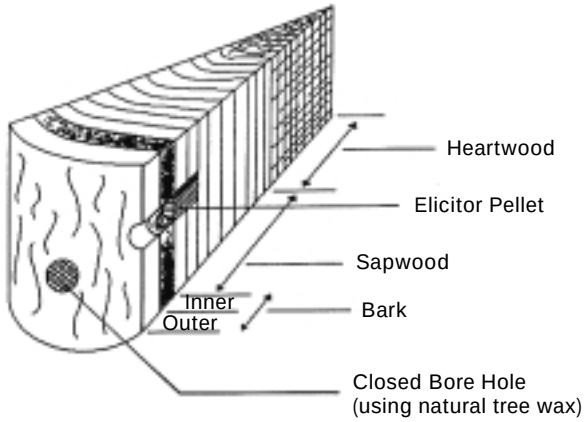

Figure 2. Schema of pellet injection procedure.

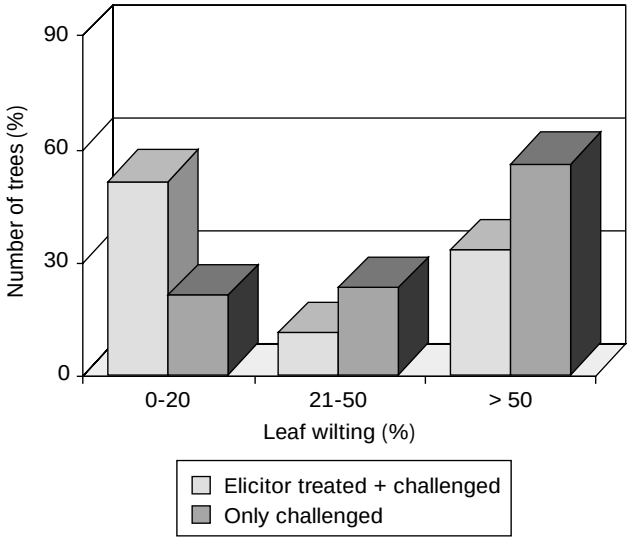

Figure 3. Leaf wilting of 4-year-old *Ulmus americana* seedlings, conditioned in Toronto with the glycoprotein elicitor and challenged 10 days later with *Ophiostoma novo-ulmi* (strain H175; 10,000 spores/seedling). Bars represent number of trees, 7 weeks after the challenging inoculation.

Elicitor tests

Preliminary greenhouse experiments with 4-year-old elm seedlings and field trees (10 cm DBH) were carried out to test their response to elicitor treatment. Injections were performed with Mauget injectors. Thin layer chromatography of branch sample extractives verified the positive reaction for mansonones induction. In subsequent nursery and field experiments gelatin plugs (Fig. 2) replaced the Mauget injectors in consideration of the high costs of the injectors. It was estimated that a viable preventive treatment should not cost more than Can$ 30 per tree. The Mauget system had the advantage of injecting the glycoprotein (elicitor) in liquid form into the tree. This provided a faster, more constant uptake and better distribution of the elicitor but appeared too expensive, since the price per injector was Can$ 2.50. A 10 cm DBH tree would require at least 4 injectors. Furthermore, prior to treatment, the elicitor had to be dissolved in distilled water. For this reason and in spite of many disadvantages of the gelatin plugs, we continued to employ them in our work. Each gelatin plug contained a designated quantity of elicitor.

To administer the gelatin plug, a 4mm bit was used to drill a hole into the youngest sapwood reaching the xylem. The gelatin pellet with or without the elicitor was inserted into the bore hole which was then closed with paraffin or wax (Fig. 2). The elicitor dosages were from 20-160 mg per tree (Table 1).

Fig. 3 summarizes the nursery results of 50 seedlings. The seedlings were treated first at their stems approximately 10 cm above ground level with the elicitor incorporated in a gelatin pellet, and challenged ten days later with 10,000 spores about 5 cm above the conditioning treatment. The disease intensity of the 50 seedlings was evaluated 7.5 weeks after inoculations with strain H175 of *Ophiostoma novo-ulmi* and classified in three categories according to their wilting symptoms: 1) from 0-20% wilting; 2) 21-50% wilting;

Table 1. Trees showing no symptoms, yellow leaves, brown leaves or dead trees in the field experiments at Kingston. Values are given in percentages. *Ulmus americana* trees were treated on May 27, 1998 and challenged with *Ophiostoma novo-ulmi* on June 11, 1998 (8,000 spores)

Date	Condition	Elicitor dosage per tree				
		0	20 mg	40 mg	80 mg	160 mg
September 10, 1998	Healthy	6	80	67	20	73
	Yellow leaves	27	13	26	47	7
	Brown leaves	67	7	7	33	20
June 9, 1999	Dead	100	33	53	60	33

and 3) over 50% wilting. The percentages of each category were 50%, 15% and 30% respectively. About **50%** of the 50 seedlings treated with the elicitor showed from 0-20% wilting of the leaves, about **15%**, showed 21-50%, and about **30%** of the seedlings showed over 50% wilting of their leaves. Corresponding figures for the water treated controls were about **20%**, **20%**, and about **50%,** respectively, and these controls showed much greater wilting than the treatment. However, there was great variation in symptom expression among the seedlings. In spite of this, the results showed that the elicitor induced resistance. The same experiment has been repeated in Northern Ontario and produced very similar results (Fig. 4).

The next step in our elicitor experiments on induced resistance were carried further out in field trials at a location near Kingston, Ontario at the forest of the Canadian Armed Forces. These experiments were started in June 1998 with 75 randomly selected trees of about 10cm in DBH. The trees were first conditioned with the elicitor and 10 days later challenged with 8,000 spores of strain H175 about 10 cm above the points of conditioning (Fig. 2). Controls trees were solely elicitor-treated.

These results, shown in Table 1, confirmed those obtained in Toronto and Northern Ontario. Great variation among the tree responses to the elicitor treatment were again obvious. These results were not surprising as the experiment was made with wild type trees of great possible genetic diversity. In these conditions, pathogen invasion would not only be affected by differences in the tree response but also by environmental conditions such as those related to soil and climatic variations. Al these factors in turn might also indirectly influence the activity of the elicitor inducing the defense reactions.

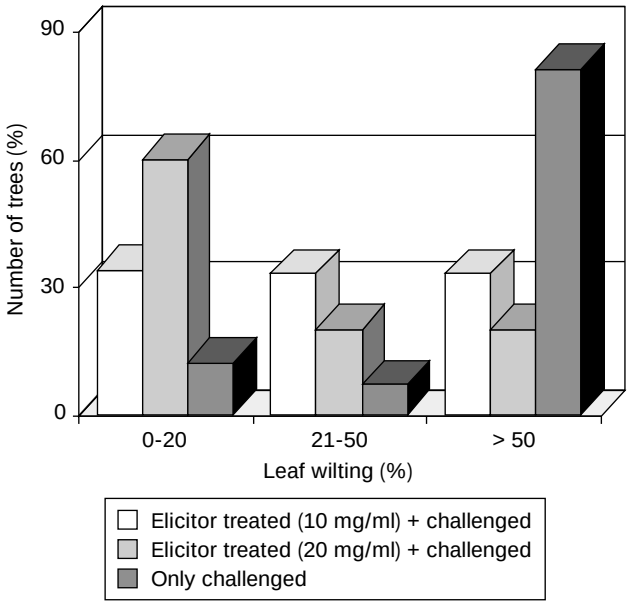

Figure 4. Leaf wilting of 6-year-old *Ulmus americana* saplings, conditioned in Northern Ontario with the glycoprotein elicitor and challenged 10 days later with *Ophiostoma novo-ulmi* (strain H175; 8,000 spores/sapling). Bars represent number of trees, 7 weeks after the challenging inoculation.

The effect of different concentrations of the elicitor on its capability to induce resistance against the highly virulent strain H175 was also tested under field conditions. Great variation in symptom expression as also in tree mortality was again evident. Contrary to predicted results the highest tree mortality was recorded with the highest elicitor dosages but not the lowest. The overall mortality however, of the 60 treated trees one year after treatment was 45% compared to 100% of the control group (15 trees).

Several factors that may have influenced the results of these experiments: 1) administration of the elicitor in the form of a gelatin pellet; 2) the point of challenge on the tree; and 3) the dosage. Disintegration of the gelatin pellet and release of the elicitor may have been very irregular depending on the sap flow of the specific tree. The point of elicitor injection might have been too close to the point of conditioning and the dosage administered too low. Production of the elicitor in great quantity at reasonable costs in large fermenters proved difficult, and did not allow treatment costs of Can. $ 30 per tree.

Induced resistance by low virulent *O. ulmi* strains in field tests with large American elms

Our first experiments on induced resistance were carried out on 4-year-old seedlings but not on large trees. Tests were required to verify if this method would protect these trees as well against DED. Consequently, preliminary field experiments were conducted on induced resistance at the Richardson property on large trees at Milton near Toronto (Hubbes, 2001).

Twenty trees between 4.7 and 10.4 cm in DHB were selected. Three trees were conditioned with 32,000 spores of the non-aggressive strain per tree and 12 others with 16,000 spores. One tree conditioned with

Table 2. Disease severity of *Ulmus americana* control trees in 2000 and 2001. Elms were challenged on a side branch with *O. novo-ulmi* on June 12, 2000 (1,000 spores)

Tree Nº	DBH (cm)	05/07/00	25/07/00	15/08/00	19/09/00	13/07/01	01/08/01	19/09/01
1	5.9	0[a]	0	0	0	4	4	4
2	5.9	0	0	2	2	3	4	4
3	5.6	0	3	4	4	3	4	4
4	8.7	0	0	0	2	0	0	0
5	4.7	0	2	3	2	4	4	4
Average		0	1	1.8	2	2.8	3.2	3.2

[a] Numbers indicate leaf wilting of: 0% (0), 10-20% (1), 21-50% (2), 51-75% (3), 100% (4).

Table 3. Disease severity of *Ulmus americana* side branches in 2000 and 2001. Elms were conditioned at breast height with *Ophiostoma ulmi* on June 1, 2000 (16,000 spores) and challenged with *O. novo-ulmi* on a side branch on June 12, 2000 (1,000 spores)

Tree Nº	DBH (cm)	05/07/00	25/07/00	15/08/00	19/09/00	13/07/01	01/08/01	19/09/01
1[a]	9.9	0[b]	0	0	4	4	4	4
2	7.3	2	0	0	3	4	4	4
3	9.1	3	1	1	1	4	4	4
4	7.6	0	0	1	0	0	0	0
5	10.5	1	1	1	4	4	4	4
Average		1.2	0.4	0.6	2.4	3.2	3.2	3.2

[a] Conditioned with 32,000 spores. [b] Numbers indicate leaf wilting of: 0% (0), 10-20% (1), 21-50% (2), 51-75% (3), 100% (4).

Table 4. Disease severity of *Ulmus americana* trees in 2000 and 2001. Elms were conditioned with *Ophiostoma ulmi* at breast height (16,000 spores) and challenged with *O. novo-ulmi* on June 12, 2000 (1,000 spores on a side branch)

Tree Nº	DBH (cm)	05/07/00	25/07/00	15/08/00	19/09/00	13/07/01	01/08/01	19/09/01
Conditioned + challenged								
1[a]	9.9	2[b]	2	1	1	0	0	0
2	7.3	2	2	3	3	4	3	4
3	9.1	2	3	2	3	4	0	2
4	7.6	1	2	2	2	0	0	2
5	10.5	1	2	2	3	0	0	0
Average		1.6	2.2	2	2.4	0.8	1	1.6
Only conditioned								
6[a]	7.9	1	1	1	1	0	0	0
7[a]	8.5	1	1	1	1	0	0	0
8	5.7	0	0	0	0	0	0	0
9	8.8	2	2	2	2	0	0	0
10	4.8	0	0	0	0	0	0	0
11	8.3	2	2	2	3	0	1	1
12	6	3	2	2	2	0	2	3
13	7.4	2	2	2	2	2	0	0
14	8.7	2	2	1	1	0	0	0
15	8.5	3	3	0	3	0	0	0
Average		1.6	1.5	1.1	1.5	0.2	0.3	0.4

[a] Conditioned with 32,000 spores. [b] Numbers indicate leaf wilting of: 0% (0), 10-20% (1), 21-50% (2), 51-75% (3), 100% (4).

32,000 spores, four trees conditioned with 16,000 spores and five non-conditioned control trees were challenge-inoculated with an aggressive strain. The strain used for conditioning was strain Q412, a non-aggressive strain of *O. ulmi,* and the aggressive challenging strain was H175 of *O. novo-ulmi*. Conditioning was performed at breast height and challenging on a side branch with 1,000 spores.

The objective of this preliminary experiment was to test whether trees treated with 16,000 or 32,000 spores of the non-aggressive strain would be killed. This strain was originally isolated from American elms showing severe DED symptoms during the first disease outbreaks in Canada. The results of these experiments are given in Tables 2, 3 and 4. Evaluation of wilting was made at 4 periods during 2000 and 3 periods during 2001 according to an arbitrary scale of severity from 0-4. The score 0 indicates no symptoms whereas score 4 indicates complete wilting. Distinction has been made between the treated branch and the whole

Table 5. Responses of *Ulmus americana* trees after conditioning at breast height with *Ophiostoma ulmi* on June 17, 2002 (16,000 spores) and challenging with *O. novo-ulmi* on June 27, 2002 (1,000 spores on a side branch)

Tree N°	DBH (cm)	Conditioning isolate	Response	Score
Conditioned + challenged				
1	29	Q412	Top dead	3
2	55	Q412	3 side branches dead, top alive	2
3	34	Q412	Inoculated branch dead	1
4	55	Q412	Inoculated branch dead	1
5	27	Q412	Inoculated branch dead	1
6	36	Q412	Inoculated branch dead	1
7	48	Q412	Dead	5
8	43	Q412	90% dead, epicormic growth	4
9	55	Q412	Inoculated branch dead	1
10	36	Q412	Inoculated branch dead	1
11	60	Q412	Inoculated branch dead	1
12	65	Q412	Inoculated branch dead	1
13	41	Q412	Inoculated branch dead	1
14	31	Q412	Inoculated branch dead	1
15	55	Q412	Inoculated branch dead	1
16	29	H5	80% dead	4
17	52	H5	Dead	5
18	46	H5	Dead	5
19	30	H5	Dead	5
20	65	H5	90% dead	4
21	57	H5	20% dead	2
22	45	H5	Dead	5
23	41	H5	Dead	5
24	60	H5	Dead	5
25	26	H5	20% dead	2
26	29	H5	20% dead	2
27	38	H5	Inoculated branch dead	1
28	30	H5	Dead	5
29	29	H5	20% dead	2
30	65	H5	Dead	5
Only challenged				
31	55	—	Dead	5
32	58	—	Dead	5
33	120	—	Dead	5
34	60	—	Dead	5
35	55	—	Dead	5

Numbers indicate leaf wilting of: 0% (0), 10% (1), 20% (2), 40% (3), 80-90% (4), 100% during the year of challenging (5).

tree (Tables 3 and 4). As seen from Table 3, all challenged side branches except that of tree #4 were dead in September 2001. In comparison, only tree #2 (Table 4) showed complete wilting on September 19, 2001, and died.

In general, trees that were first conditioned and then challenged and those that were conditioned only showed in the first year somewhat higher disease symptoms than the controls trees (Table 4). Most of the trees that were only conditioned did not show any leaf symptoms the year after conditioning. There appears to be no difference in leaf symptoms between trees conditioned with 16,000 or 32,000 spores of strain Q412; all trees survived one year after treatment

In contrast, 4 out of 5 control trees that were only challenged with the aggressive strain were dead in September 2001 (Table 2). A similar experiment was repeated in 2002 with two non aggressive strains to test whether differences exist among non-aggressive strains in their potential to induce resistance (Table 5).

The 2002 field experiments were conducted in a randomized format. One additional score point, 5, was added for denoting trees which died during the current year in consequence of treatment. The results in Table 5 show that, in comparison to the control, both strains induce resistance. Nevertheless, the protection rate by Q412 ranged in the 87% whereas protection by H5 ranged in the 33%.Results with these field tests with H5 do not concur with those obtained in preliminary inoculation experiments with 4-year-old American seedlings. Beside its great potential to induce resistance, isolate Q412 presents another interesting aspect as already noticed in the previous inoculation experiments. The inoculated side branch died back in most cases. Presently, we do not know whether this is also the case in multiple side branch inoculations. Further experiments will clarify this. Furthermore, it needs to be established whether the aggressive strain is stopped at the basis of the branch by the trees defense or whether the fungus penetrates the main stem and is prevented from further spread. Investigations are underway to elucidate this.

Conclusions

Induced resistance to control Dutch elm disease is environmentally friendly it is linked to the defense response of the tree. Dutch elm disease can be controlled by induced resistance either by conditioning the tree with a glycoprotein isolated from the fungus or by a low virulent strain of *O. ulmi*. The efficacy associated with induced resistance appears to be strain dependent and presently the use of a specific non-aggressive strain of *O. ulmi* to reach this end is more cost efficient. Conditioning with non-aggressive strains is not dose dependent since their distribution is maintained by continuous growth of the fungus in the tree vascular system. The biochemical basis of trees defense is not well understood and requires further investigations. Only by a full understanding of the molecular genetic basis of the tree defense mechanisms can the typical umbrella-shaped American elm be protected from the attack normally virulent strains of *O. novo- ulmi*.

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