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Canada Yew: Developing a Value-Added Crop for Northern Ontario

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Abstract

Canada yew (*Taxus canadensis* Marsh.) is a native evergreen shrub found in most of Ontario that contains anticancer compounds called taxanes in its needles, bark, and roots. In 2004, a research project was initiated to (i) develop methods for selecting individual yew plants with high growth rates and high taxane concentrations, and (ii) determine the best methods for growing Canada yew in plantations, towards the goal of developing the capacity to produce a commercially viable yew crop in northern Ontario. Four plantations were established in the Algoma district of Ontario: one in June 2004 at the Ontario Forest Research Institute (OFRI) arboretum and three in late August 2005 at the OFRI arboretum, Thessalon First Nation BioCentre, and Whelan farm. Treatments imposed on all the plantations were (1) crop plant spacing at 30 and 45 cm, (2) soil treatments of mulch, fertilization, and mulch-plus-fertilization. Compost and mulch-plus-compost treatments were applied only on the 2004 plantation. Compared to the controls, only the 2004 plantation showed significant treatment-related increases in growth in all but the spacing treatments, with fertilization and mulch-plus-compost resulting in about 15% to 50% more growth, respectively, than the other treatments. Fertilization provided the most cost effective increase in yew plantation growth. Deer browsed the plants at all sites and, of the three herbivore control measures attempted, only the electric fence successfully excluded deer. Selection of individual yew plants for high growth rates and taxane concentrations yielded individuals with 3 to 4 times the potential taxane production capacity of the average plant. Further development of Canada yew as a value-added crop will require these high taxane-producing individuals to be propagated for plantation culture.

Resumé

L'if du Canada (Taxus canadensis Marsh.) est un arbrisseau indigène persistant dont les aiguilles, l'écorce et les racines ont une forte teneur de composés chimiques anticancéreux appelés taxanes. L'if du Canada se retrouve dans la plupart des régions de l'Ontario. En 2004, un projet de recherche a été lancé 1) afin de développer des méthodes de sélection d'arbres individuels présentant des taux de croissance élevés et des concentrations élevées de taxanes, et 2) afin de déterminer les meilleures méthodes à utiliser pour faire pousser l'if du Canada dans des plantations, dans le but de développer la capacité de produire, dans le Nord de l'Ontario, des récoltes d'if qui soient commercialement viables. Quatre plantations ont été crées dans le district d'Algoma de l'Ontario : l'une en juin 2004 à l'arboretum de l'Institut de recherche forestière de l'Ontario (IRFO), et trois à la fin d'août 2005 à l'arboretum de l'IRFO, au biocentre des Premières nations de Thessalon et dans une pépinière de Whelan. Toutes les plantations ont reçu les traitements suivants : 1) espacement des plants de 30 et de 45 cm, 2) traitements du sol à l'aide de paillis, de fertilisants et d'une combinaison de paillis et de fertilisants. Les traitements à l'aide de compost et d'une combinaison de compost et de paillis ont été appliqués seulement aux arbres plantés en 2004. La comparaison avec les données de contrôle révèle que seuls les arbres plantés en 2004 affichent une croissance importante attribuable aux traitements dans tous les traitements sauf l'espacement des semis, et que la croissance des arbres traités à l'aide de fertilisants et d'une combinaison de paillis et de compost était supérieure 15 % à 50 % respectivement à celle des arbres qui ont reçu les autres traitements. La fertilisation s'est avérée la méthode la moins coûteuse pour augmenter la croissance des ifs plantés. À tous les endroits, les chevreuils ont brouté les jeunes pousses, et de toutes les mesures de contrôle des herbivores mises à l'essai, seules les clôtures électriques ont réussi à éloigner les chevreuils. Les plants sélectionnés pour leur taux de croissance élevé et leurs concentrations élevées de taxanes ont produit des arbres dont la production de taxanes serait de 3 à 4 fois plus élevée que celle des arbres moyens. Pour développer la culture à valeur ajoutée de l'if du Canada, il faudra propager les semis ayant de grandes quantités de taxanes et les cultiver dans des plantations.

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Introduction

In a 1962 bioprospecting campaign for the National Cancer Institute, botanist Arthur Barklay collected about 15 pounds of Pacific yew (*Taxus brevifolia*) bark, needles, and twigs (Stephenson 2002). These samples were analyzed by Dr. M. Wall in North Carolina and extracts of these tissues, especially the bark, showed promise as an anticancer agent. By 1971, Wall and Dr. M. C. Wani of the Research Triangle Institute in North Carolina had purified, identified, and reported the chemical structure of the complex molecule they named taxol, as the active anti-tumour chemical from the Pacific yew bark (Wani et al. 1971). However, it required all the bark of one mature 12 m Pacific yew tree to produce half a gram of taxol and since yew trees are infrequent in the coastal forest of the Pacific Northwest, taxol production was hampered by bark supply problems. Additional extraction and processing problems prevented the production of taxol of sufficient purity.

Dr. Susan Horwitz identified the mode of action of taxol as preventing cell division (Schiff et al. 1979). Taxol's unique mechanism of action was to stabilize proteins called tubulins in dividing cells. Microtubules, which are made from tubulins, are structures formed during cell division that push the two sets of newly replicated chromosomes to separate sides of the cell. By binding to the microtubules, taxol inhibits microtubule disassembly, thereby halting cell division. This discovery led the National Cancer Institute (NCI) to begin clinical trials in the United States in 1983. By 1989, these trials had shown sufficient promise in treating ovarian cancer for NCI to request that pharmaceutical companies commercially develop taxol and seek to alleviate supply shortfalls.

Bristol Myers Squibb (BMS) was awarded the contract to develop taxol and in 1992 received Food and Drug Administration (FDA) approval to market it as a treatment for ovarian cancer (Stephenson 2002). In 1994, BMS registered Taxol® as its trademark name for the chemotherapy drug and assigned paclitaxel as the generic chemical name. Difficulties in harvesting enough yew bark limited the initial supply of the drug and in late 1993 the price spiked to US$5,846 per gram. BMS had anticipated the shortage and in 1990 had contracted research chemist Dr. Robert Holton of Florida State University to solve the supply problem. He developed an efficient procedure (80% minimum conversion rate) that used a paclitaxel precursor, 10-deacetylbaccatin III (10-DAB), from English yew (*Taxus baccata*) as a semi-synthetic source of paclitaxel and docetaxel (Holton 1993). In 1995, BMS received FDA approval to use this semi-synthesis process to produce paclitaxel. Since 1992 Taxol® has also been FDA approved to treat a number of other cancers such as breast, non-small cell lung, and AIDS-related Kaposi's sarcoma.

Despite its 5-year marketing exclusivity agreement with the FDA that ended in 1997, BMS used its questionable patents to legally fend off generic manufacturers, and its sales of Taxol® grew by 38% per year, peaking in 2000 at US$1.6 billion (Stephenson 2002). Lawsuits filed by Ivax Ltd., an American generic drug company, and Biolyse Pharma, a Canadian generic drug company, challenged the BMS patents and ended its Taxol® exclusivity in the United States in 2002 and in Canada in 2005. Manufacturing competition from a number of generic drug companies resulted in price decreases and they are now hovering at about US$100 per gram or less for the purified paclitaxel powder which is sold to pharmaceutical companies. Total worldwide annual sales (2006) of the drug form were just over US$1 billion (Conlin et al. 2007). In addition, improved second-generation taxane-derived drugs such as Taxotere® and Abraxane® have been approved as chemotherapy drugs since 1992. Finally, a number of experimental taxane derivatives touted to be up to 1000 times more effective against cancer than paclitaxel (based on animal trials) are now in stage I and II human clinical trials at Holton's spinoff company, Taxolog Inc., and others (Stephenson 2002, Ferlini et al. 2008). These new and potential future chemotherapy drugs will likely continue to drive increased demand for taxanes from plant biomass since all use the taxane compounds extracted from yew plants as their base.

The use of English yew as a source of paclitaxel and 10-DAB led to the investigation of all yews (*Taxus*) for taxane content. Canada yew (*Taxus canadensis* Marsh) was discovered to be a good source of paclitaxel as well as two paclitaxel precursors, 10-DAB and 13-acetyl-9-dihydrobaccatin (DHB). The latter is a compound unique to Canada yew (Zamir et al. 1995). One reason that Canada yew is of commercial interest is its relatively high concentrations of taxanes compared with other species of yew (Cass et al. 1999, van Rozendaal et al. 2000). Another reason is that paclitaxel can be semi-synthesized from DHB (Nikolakakis et al. 2000), which occurs at 5 to 7 times the concentration of paclitaxel in Canada yew (Zamir et al. 1995) although others (Cameron and Smith 2008) have reported DHB averages only 3 times the paclitaxel level. Also of note is that the 10:1 ratio of 10-DAB to paclitaxel reported by van Rozendaal et al. (2000) is much higher than the 1:1 ratios reported by Cass et al. (1999) and Cameron and Smith (2008) for Canada yew.

Cultivars of English yew selected for fast growth and high taxane concentrations (Wang et al. 2006) are grown in plantations and used as a major biomass source for taxanes produced by the pharmaceutical industry. Since Canada yew plants have been found to have higher taxane concentrations than English yew (van Rozendaal et al. 2000) it should be possible to select Canada yew individuals that are more efficient at producing taxanes, particularly paclitaxel, than English yew. Growth rates of Pacific yew vary significantly depending on site characteristics (Bailey and Liegel 1998) and Canada yew growth has been shown to respond to differing conditions in the forest and plantations (Campbell et al. 1998, Smith et al. 2006) suggesting that environmental conditions for its growth can be optimized. Canada yew has been found to have varying levels of taxanes in its tissues with the highest concentrations found in the needles (Cass et al. 1999, van Rozendaal et al. 2000). Daily and seasonal variations in taxane levels have been reported for English yew cultivars (ElSohly et al. 1997) and Canada yew (Cameron and Smith 2008). Selection of elite individual Canada yew plants for higher growth rates would increase the amount of biomass produced annually, and selection for higher paclitaxel concentration would not only increase the yield per hectare, but improve subsequent extraction and purification efficiencies as well. Propagation of selected elite individuals and introduction of these individuals to a farm or nursery-based plantation system could help produce more paclitaxel at a lower price and promote a new industry for Northern Ontario. Therefore, plantations of these elite plants will provide future growers the potential to add value to their Canada yew plantations making them more profitable (Robertson 2005).

To facilitate more efficient production of paclitaxel, the objectives of this research study were:

(1) To attempt to develop a northern Ontario source for cutting-propagated Canada yew plants.
(2) To test approaches to optimize Canada yew growth and develop its potential as a value-added crop for northern Ontario farmers.
(3) To determine if paclitaxel concentrations in Canada yew foliage can be maximized by:
    a) selecting individuals with high growth rates and paclitaxel concentrations
    b) determining optimal growing conditions
    c) determining optimum harvest time

Materials and Methods

This research project comprised two main studies and a smaller third study. First, a crop plant selection study was used to select individual yew plants with the greatest potential for taxane production ('elite' plants). Second, a plantation culture study was begun to determine the best soil type, soil treatments, and plant spacing to most efficiently grow yew and yield the highest taxane concentrations. Third, a herbicide study was established to determine the effects of two pre-emergent herbicides on yew plant growth.

Crop Plant Selection Study

In 2004, cuttings from 296 individual Canada yew plants were collected from 3 regions of Ontario at different times: (1) Algoma (east shore of lake Superior and north shore of Lake Huron up to Elliot Lake) in May, (2) the northwest (north and west of Marathon, ON) in August and (3) the northeast and central (from Hearst south and east to Huntsville area) in September. Because of the clonal nature of yew (Corradini et al. 2002), two plants (6 cuttings each) usually separated by a minimum of 5 m were sampled from each site with a minimum of 5 km separating most sites. Cuttings from each plant were placed in plastic bags and stored in a cooler with ice. Upon return to the lab, cuttings were stored at +2°C until propagated, with a maximum of 14 days in cold storage between harvest and propagation.

Cuttings were rooted using procedures developed by the Canadian Forest Service (Yeates et al. 2005). Briefly, this method involved cutting a 12 to 18 cm section representing one or two year's growth from the terminal portion of the branch at the node between it and the previous year's growth, stripping the foliage and side branches from the lower half, dipping the cut end into water, coating it with Stim-Root® #3 rooting powder containing 0.8% indole butyric acid, and placing it into a cavity of a Multipot 67 reforestation container containing a 2:1 mix of moistened peat moss and perlite. The Multipots were then placed in a greenhouse with misters set to maintain the relative humidity at a minimum of 70%, a 16-hour photoperiod supplemented with sodium vapour lamps during cloudy periods or short days, and a temperature regime of 23°C days and 17°C nights. When sunlight intensity exceeded 1800 umol photons m^{-2} sec^{-1} (400 W m^{-2}), shade curtains (50% shading) automatically closed. Periodic visual surveys were used to assess the number of cuttings with roots.

On July 13, 2005, these yew cuttings were transferred to an outdoor holding area and placed under one layer of 50% shade cloth until November 17, 2005 when they were transferred back to the greenhouse at a constant 5°C and natural day length (no supplemental lighting). Foliage samples for taxane analysis were collected from most individual plants (some lacked sufficient foliage) in December. On January 4, 2006, the growing conditions were changed to a 14-hour day with supplemental sodium vapour lighting and 22°C day and 15°C night temperatures to induce bud burst and new shoot growth. Shoot growth started in late January and virtually all plants had set bud by March 15, 2006 when growth measurements of the number and length of new shoots started. On March 26, 2006 growing conditions were changed to an 11-hour photoperiod with 20°C days and 10°C nights to harden plants for outplanting. On June 13 and 14, individual yew plants were planted into slits cut at 0.5 m spacing in landscape cloth at the Ontario Forest Research Institute (OFRI) arboretum. About 5 to 6 cm of pine bark mulch was spread over the landscape cloth after planting. In November 2006, foliage samples were collected from most plants (some were too small to provide sufficient foliage) for taxane analysis.

In June 2007, the plants were fertilized at 81 kg ha^{-1} of 16-16-16 fertilizer. In August, survival was determined and new shoot growth (number and length of new shoots) was measured. By multiplying the total taxane (paclitaxel, 10-DAB, plus 9-DHB) concentration of the foliage (November 2006 value) by the length of new shoot growth (2007 value) and dividing this value by 5000 (an arbitrary number used to produce a numerically reasonable index value), a taxane production index for individual plants was created to rate the taxane production of the individual plants. Since more than one cutting from each plant was usually available for growth measurements, the mean growth of all surviving cuttings from a single plant was used for the new shoot growth value.

Plantation Culture Study

On June 9, 2004, 9600 yew plants from the Canadian Forest Service (CFS) Atlantic Forestry Centre in Fredericton, New Brunswick, were planted at the OFRI arboretum on a loamy soil site that had been prepared with glyphosate (2 kg active ingredient (a.i.) ha^{-1}), ploughed and disked, and then treated with Simadex (3 kg a.i. ha^{-1}) herbicide. Cuttings for these plants were propagated using the method of (Yeates et al. 2005). Two hundred 20-month-old cuttings were planted in each of 48 blocks. Each block had 3 adjacent rows with half of the cuttings planted at 30 cm and the other half at 45 cm spacing, for a total of 100 plants per spacing. This plantation is referred to as the CFS plantation throughout the remainder of this report.

In May 2005, soil treatments were applied to this plantation as follows: control (no fertilizer), fertilizer, mulch, compost, mulch-plus-fertilizer, and mulch-plus-compost, with each treatment applied to 8 blocks. Fertilizer was applied at a rate of 68 kg ha^{-1} of 16-16-16 in 2005, but in 2006 and 2007 the rate was increased to 940 kg ha^{-1} split into two applications in June and July. Mulch consisted of a one-time treatment of pine bark (5 to 7 cm thick) and compost was a one-time application of dry, screened compost (4 to 5 cm thick, from Lemieux Composting and Haul Away, Sault Ste. Marie, ON). In the combination treatment compost plus-mulch was applied before the mulch. In 2005, weed control was performed manually within the blocks and by tilling between the blocks. Manual weed control, tilling, and glyphosate applications (2 kg a.i. ha^{-1} with yew plants covered with plastic pots) were carried out in 2006 and 2007. Due to heavy deer browse on more than 90% of the CFS plantation yew plants in the spring of 2005, a 2.5-m-high Electrobraid® electric fence (Yarmouth, NS) was installed around the site in August.

Between August 8 and September 1, 2004, 28,900 cuttings were collected from 6 areas near the north shore of Lake Huron (Table 1) by project partner Forest and Land Control Inc. All cuttings were delivered to the Thessalon First Nation BioCentre within two days of harvest and stored at +5°C until propagated. Propagation occurred within 7 days of delivery.

Table 1. *Locations and dates of collection of Canada yew plantation cuttings used in this study.*

Collection location	Site Latitude and Longitude	Date collected
Dunlop Township	46° 29' 9.6" N, 82° 42' 7.0" W	August 8, 16
Dubourne Lake area	46° 14' 3.4" N, 82° 55' 55.0" W	August 17
Basswood Lake area	46° 22' 43.2" N, 83° 22' 2.8" W	August 18
Serpent River Township	46° 13' 16" N, 82° 24' 47" W	August 21, 22
Boychuck Township	46° 28' 44.8" N, 82° 39' 9.0" W	August 23, 24
Cranberry Township	46° 15' 43.8" N, 82° 54' 38.8" W	Aug. 25, 26, Sep. 1

Propagation method was as described in Yeates et al. (2005) with one exception: the premoistened media used to fill the Multipot 67s was a 1:1 mix of peat moss and perlite. Greenhouse rooting conditions were similar to those employed in the individual plant procedure with the following exceptions: At the BioCentre, shade cloth was used continuously and cuttings were hardened with a natural photoperiod beginning December 1. On December 22 cuttings were moved to cold storage (5°C) where they were watered as needed but were not misted and had an 8-hour photoperiod provided by sodium vapour lamps. On March 20, 2005, cuttings were moved back to the propagation greenhouse with 16-hour supplemental light. Rooting surveys were done pre- and post-cold storage by examining 10% of all cuttings for root formation. Yew plants remained under the 16-hour photoperiod until August 1 when supplemental lighting was withdrawn and watering reduced to induce bud set and plant hardening in preparation for extraction and planting.

Rooted yew cuttings propagated at the Thessalon BioCentre were extracted from the Multipot 67s in late August, organized in bundles of 25, wrapped with plastic, and placed in waxed boxes for distribution. On August 29 they were transported to three plantation locations: the loam soil site (OFRI arboretum), the clay soil site (Whelan Farm), and the sandy soil site (Thessalon BioCentre). From each location fifty plants were randomly selected for paclitaxel analysis and pre-plant morphological measurements. Planting was completed by August 30 at the loam site, September 2 at the clay site, and September 6 at the sandy site (Table 2). Two hundred plants were established in each block of 3 rows at each site, half at 30 cm spacing and half at 45 cm spacing. Fertilizer, mulch, and mulch-plus-fertilizer treatments were applied in summer 2006 to 7 or 8 blocks at each site. Mulch was applied as a 5 to 7 cm layer. Fertilizer was applied as 16-16-16 at 200 kg ha^{-1} in June 2006 and 2007. Weeds were controlled manually at all three plantation sites and applied to all treatments, including the control plots, but not within the plots that formed part of the herbicide trial (described in the next section). At the OFRI arboretum only, some glyphosate (with plants covered) was used for additional weed control and plantations were irrigated in all growing seasons. At the sandy site, plots were irrigated only in 2006. At the clay site no irrigation occurred during 2006 but a drip irrigation system installed in June 2007 was used throughout that growing season. Yew survival was enumerated each summer and at the end of the study.

Table 2. *Locations (and soil types), establishment dates, number and source of plants, and soil treatments applied to Canada yew plantations in this study.*

Location (soil type)	Date planted	Number planted	Plant source	Soil Treatments Applied
OFRI arboretum (loam site)	June 2004	9600	Canadian Forest Service	control, fertilizer, mulch, mulch+fertilizer, compost, mulch+compost
	Aug. 2005	8000	Thessalon BioCentre	control, fertilizer, mulch, mulch+fertilizer
BioCentre (sandy site)	Aug.-Sept. 2005	8100		control, fertilizer, mulch, mulch+fertilizer
Whelan Farm (clay site)	Aug.-Sept. 2005	7400		control, fertilizer, mulch, mulch+fertilizer

Due to deer browsing of crop plants at the sandy and clay sites, floating row covers were applied to the sandy plantation to protect it from browsing from fall through early spring. However, these row covers twice washed off in heavy fall rains so large lathe shade covers (Figure 1) were placed over the yew blocks in early October and removed in late May after the seedlings had flushed. At the clay site, a free-roaming dog was used to deter deer browsing. Manual weed control was used at both these sites.

Figure 1. *At the sandy site, lathe shade covers were installed seasonally to deter deer browsing.*

In October 2006, three randomly chosen whole plants from each spacing treatment of each block were harvested by hand from the CFS plantation. Whole plants were harvested at the loam and sandy sites in October 2007. Only new shoots (simulating a hedging or partial shoot removal type harvest) were harvested from the plantation at the clay site in October 2007. At the loam site whole plants were removed using a mechanical lifter (Figure 2) with a shaking bar that undercut the plants at a depth of about 15 cm and loosened the plants from the soil. The roots were then rinsed in water. At the sandy site whole plants were dug up by hand. At the clay site hand clippers were used to harvest only the new shoots. Plants or shoots were placed in woven plastic bags, labelled, and sealed, and the bags transported to OFRI for storage at 2°C until processed.

Foliage samples (10 g fresh weight with equal amounts of current and year-old foliage) for taxane analysis were taken from randomly chosen plants from each spacing (5 g from each spacing) of each block (48 total) of the CFS plantation and stored at -20°C until analysis.Plants were washed at a root wash station (Fawcett and Paterson 1994), separated into roots and shoots, weighed separately, dried in an oven at 70°C for 48 hours and then weighed again to determine the dry weight.

Figure 2. Mechanical lifting of Canada yew plants at the OFRI arboretum.

Herbicide Study

At the OFRI arboretum, seven blocks planted in August 2005 were reserved for herbicide trials and no soil treatments were applied. The Princep® (pre-emergent simazine herbicide) trial was established in fall 2006 in one yew block (200 plants). Glyphosate (at 2 kg a.i. ha^{-1}) was applied (plants were covered with plastic pots) on September 14, 2006 to eliminate existing competition. Prior to the Princep® treatment, dead biomass was removed from the block and the block was separated into two treatments: half the plants were covered during application and the other half were subject to the herbicide spray. The block was sprayed on October 6 at a rate of 3 kg a.i. ha^{-1}. Plants were harvested in October 2007 and processed as described for the plantation culture study.

The Goal® (pre-emergent oxyfluorfen herbicide) trial began with an application (plants were covered with plastic) of glyphosate (2 kg a.i. ha^{-1}) on October 6, 2006 to kill existing weed competition. After removal of dead biomass, Goal was applied on May 9, 2007 in each of 5 blocks at: zero (control), 0.25, 0.5, and 1.0 kg a.i. ha^{-1}, with approximately 20 plants per block treated at each concentration. Yew plants were assessed visually for damage (rated 1-4 for foliage appearance with 4=green and 1= yellow/brown) and survival on May 28 and 31. New shoot growth of all plants was measured on September 5 and 6. For shoot growth measurements the primary shoot (1°) was defined as the longest terminal shoot of the plant, secondary shoots (2°) branched off the 1° shoot, and tertiary shoots (3°) branched off the 2° shoots. Plants were harvested in October and processed as described for the plantation culture study.

Taxane Analysis

Canada yew shoots were sampled December 7-9, 2005 and October 31 and November 2, 2006 from crop plant selection study plants (current year) and October 2, 2007 from CFS plantation culture study plants (current and 1 year old) and placed in -20°C frozen storage until analysis. Shoots were air dried at room temperature (22-24°C) for 30 minutes. The shoots were cut up and oven dried at 50-55°C for 24 hours. Foliage samples (0.1 to 1 g fresh weight or 0.05 to 0.5 g dry weight) were extracted at room temperature, by homogenizing the needles in 10 ml of 100% MeOH and steeping them for 24 h in the dark. The solvent was decanted and filtered using pre-prepared tubes lined with filtering frits, attached to a Visiprep SPE manifold (Supelco). The methanolic extracts were evaporated in a SpeedVac to obtain a crude extract. Each 0.1 to 1.0 g of needles (fresh weight) yielded 16 to 160 mg of extract.

An Agilent 1200 high performance liquid chromatograph (HPLC) equipped with a computer, a binary pump, an autosampler, and an autoscan photodiode array detector was augmented by a Curosil-PFP Phenomenex (250 x 4.60 mm i.d.) analytical column. A modified gradient chromatographic technique (Phenomenex) was used at room temperature with an acetonitrile/water solvent system. Samples were eluted using a 25/75 to 65/35 gradient of acetonitrile/water over a 40-minute period with a flow rate of 1.0 ml/min. Compounds were detected at a wavelength of 228 nm and resolved peaks were scanned by the photodiode array detector from 200 to 400 nm. A dilute solution (10 mg/mL) of extract was filtered through 13 mm GHP 0.45µm Minispike (Waters, EDGE) and 10 µL injected onto an HPLC column. Peaks were identified on the basis of retention times and ultraviolet spectra. Peak areas, measured as absorbance at 228 nm, were converted to mg/ml using conversion factors obtained from external taxane standards (ChromaDex Inc., 2952 S. Daimler St., Santa Ana, CA USA. Paclitaxel, 10-deacetylbaccatin III, 13-acetyl-9-dihydrobaccatin III, cephalomannine, baccatin III, dehydroxybaccatin, 10-deacetyltaxol, 10-deacetylcephalomannine, and 7-epi taxol).

Data Analysis

Data were analyzed using SigmaStat for Windows (ver. 3.0) general linear model one-way analysis of variance; the Holm-Sidak method was used to compare differences between means ($p \leq 0.05$). Non-normally distributed data were transformed using square root or natural log functions. If this did not result in normal distributions, nonparametric analysis was performed using Kruskal-Wallis analysis of variance on ranks and Dunn's test for comparing means ($p \leq 0.05$).

Results

Plantation Culture Study

Only results from the 2004 CFS plantation will be presented in this section. The confounding influence of the variable but unmeasured levels of deer browsing on yew growth at each of the three sites planted in 2005 compromised the growth data. These results are provided in Appendix 1.

Survival

Survival varied by treatment (Table 3) and was highest in the compost, mulch-plus-compost, and mulch-plus-fertilizer treatments. Plants in the mulch or fertilizer alone treatments had lower survival and those in the control treatment had the lowest survival. However, it should be noted that deer browsing likely had a confounding effect on survival.

Table 3. *Final survival of yew plants in 2004 CFS plantation at the OFRI arboretum. Treatment means followed by the same letter are not significantly different ($p \leq 0.05$).*

Treatment	Yew survival (%)
Control	21.7 c
Mulch	46.8 b
Fertilizer	51.8 b
Mulch+Fertilizer	65.3 a
Compost	59.9 a
Mulch+Compost	59.8 a
Overall	52.0

Plantation Growth

After 4 growing seasons, all treatments significantly improved final growth of the yew plants in the 2004 CFS plantation compared to those in the controls (Table 4). Mulch-plus-compost treatments increased overall plant growth to the greatest extent, and root growth in particular. Fertilizer treatment produced the next best growth response, with shoot growth showing the greatest increases over control plants compared to any other treatment. Similar results were observed in the 2006 harvest of the 2004 CFS plantation (data not shown). In the 2006 and 2007 harvests, plant spacing did not affect growth: in the 2007 harvest overall mean plant dry weight for plants established at 30 cm spacing averaged 18.2 g and for plants established at 45 cm averaged 17.1 g.

Shoot-to-root ratios were significantly affected by the different treatments (Table 4). Yew plants in the mulch-plus-fertilizer and mulch-plus-compost treatments had the lowest shoot-to-root ratios, while those in the mulch and compost treatments had mid-range ratios, and those in the fertilizer and control treatments had the highest ratios. Treatments had more effect on root growth than shoot growth.

Table 4. Effect of soil treatments on total yew biomass and shoot:root ratios at the 2004 CFS plantation harvested in October 2007. Within a column, means (n=840 plants) followed by the same letter are not significantly different (p ≤0.05).

Treatment	Biomass (g DW) per plant			Shoot:root ratio
	shoot	root	total	
Control	6.54 d	2.67 c	9.21 d	2.48 a
Fertilizer	14.38 b	5.70 b	20.08 b	2.52 a
Mulch	11.79 c	5.43 b	17.23 bc	2.19 b
Compost	11.06 c	5.11 b	16.17 c	2.16 b
Mulch+Fertilizer	11.00 c	5.94 b	16.94 bc	1.86 c
Mulch+Compost	17.49 a	8.82 a	26.31 a	1.97 c

Fourth-year (Figure 3) and proportional growth (Figure 4) of yew plants were significantly influenced by treatments. The mulch-plus-compost treatment increased growth the most. In addition, plants grown with mulch, mulch-plus-fertilizer, and fertilizer grew more than those in the control or those with compost. Plants in the compost treatment were the only ones that did not outgrow untreated control plants. On average, all of the plants in the mulched treatments had fourth-year proportional growth increases 1.5 times greater than those of controls, whereas other treatments increased plant growth by a factor less than one. This suggests that all of the mulched treatments have significantly higher growth rates at this stage of growth than plants in other treatments.

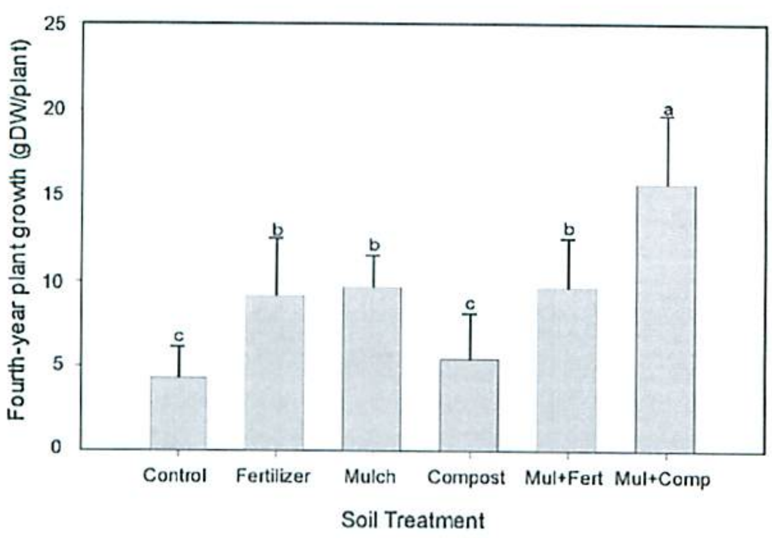

***Figure 3.** Effects of soil treatments on fourth-year total plant growth in the 2004 CFS yew plantation. Error bars = 1 standard error; bars topped by a different letter are significantly different (p ≤0.05).*

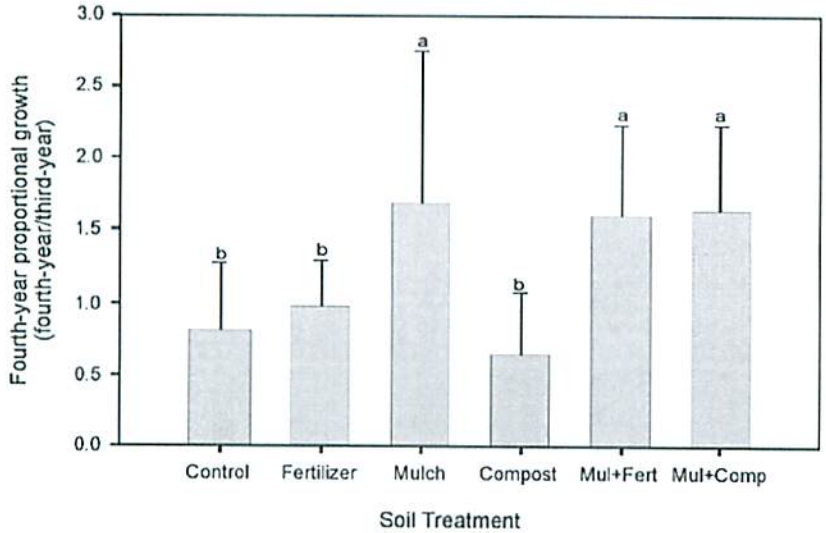

Figure 4. Effects of soil treatments on proportional yew growth (based on the ratio of fourth year new growth to third year total plant biomass) in the 2004 CFS plantation. Error bars = 1 standard error; bars topped by a different letter are significantly different ($p \leq 0.05$).

Herbicide Study

Shoot growth was somewhat affected by the application of Princep® but not by Goal® herbicide (Table 5). Total harvested biomass and shoot-to-root ratios were not affected by either herbicide (Table 6). Plants sprayed directly with Princep® on average had 3 fewer new shoots and less tertiary shoot growth than did those protected from direct spray. Goal® had no significant effects on shoot growth. However, in both trials the foliage exposed to herbicides was chlorotic compared to foliage of control plants. Although both pre-emergence herbicides limited new weed growth, and especially the rate of growth, to some extent, significant new weed growth nonetheless occurred in all the sprayed plots. Visual assessments on May 31, 2007 revealed no significant effects of Goal® on yew foliage condition but weed control was significantly better in the plots treated with 1.0 kg a.i. ha^{-1} than in the control plots (data not shown).

Table 5. Princep® and Goal® herbicide trial results: yew shoot growth measured September 2007. Within a column for a single herbicide, means (n=50 plants for Goal trial, n=80 plants for Princep trial) followed by the same letter are not significantly different ($p \leq 0.05$).

Treatment	New shoot growth (mm)				New shoots (#)
	Total	1°*	2°	3°	
Princep control	217 a	37 a	159 a	21 a	11 a
Princep exposed	173 a	34 a	127 a	12 b	8 b
Goal control	233 a	46 a	162 a	25 a	10 a
Goal 0.25 kg a.i. ha^{-1}	227 a	41 a	163 a	23 a	10 a
Goal 0.50 kg a.i. ha^{-1}	160 a	31 a	112 a	16 a	8 a
Goal 1.00 kg a.i. ha^{-1}	171 a	34 a	121 a	16 a	9 a

* Definitions for 1°, 2°, 3° shoots provided in methods.

Table 6. Princep® and Goal® herbicide trial biomass growth results: yew harvested fall 2007. Within a column for a single herbicide, means (n=50 for Goal trial, n=80 for Princep trial) for a single trial followed by the same letter are not significantly different (p ≤0.05).

Treatment	Biomass (g DW) per plant			Shoot:root ratio
	shoot	root	total	
Princep control	1.16	0.71	1.87	1.63
Princep exposed	1.13	0.67	1.80	1.69
Goal control	2.07 a	0.85 a	2.92 a	2.37 a
Goal 0.25 kg a.i. ha^{-1}	1.75 a	0.77 a	2.52 a	2.28 a
Goal 0.50 kg a.i. ha^{-1}	1.54 a	0.65 a	2.19 a	2.42 a
Goal 1.00 kg a.i. ha^{-1}	1.58 a	0.67 a	2.25 a	2.38 a

Crop Plant Selection Study

Individually propagated yew plants exhibited variation in rooting efficiency (Table 7) and growth characteristics in the greenhouse and the field (Table 8). For example, in the greenhouse maximum shoot growth was 20 times the minimum. The best shoot growth in the greenhouse was about twice that of average growth. Field shoot growth showed similar trends with an even larger range from minimum to maximum growth and a maximum shoot growth of about three times the average. Similar trends were evident in the number of new shoots.

Table 7. Propagation efficiencies of cuttings from individual yew plants grown in the greenhouse.

Collection region	Plants collected (#)	Rooting efficiency (%)
Algoma	106	65
Northeast/Central	152	52
Northwest	38	75
Total	296	60

Table 8. Shoot growth characteristics of individual yew plants (n≈1000 plants) grown in the greenhouse compared to those grown in the field.

Shoot growth parameter		2005 greenhouse	2007 field
Total new shoot length (mm)	Minimum	36	20
	Average	373	955
	Maximum	831	2957
Number of new shoots	Minimum	1	1
	Average	11	30
	Maximum	28	75

Selection of Individual Yew Plants for Taxane Concentration

Taxane concentrations of individually propagated yew plants varied widely (Table 9). The range from highest to lowest paclitaxel concentration was about 100 fold in 2005 and about 40 fold in 2006. Average paclitaxel concentration was 6 and 5 times lower than the highest concentration in 2005 and 2006, respectively. Perhaps more importantly, maximum total taxane concentration in 2006 was more than 3 times higher than average total taxane concentration and over 35 times higher than the minimum concentration.

Table 9. *Paclitaxel and taxane concentrations (µg/g dry weight) of individual yew plants sampled fall 2005 and 2006.*

Concentration/ samples	Paclitaxel 2005	Paclitaxel 2006	10-DAB 2006	DHB 2006	Total taxanes 2006
Minimum	4	10	10	208	261
Average	83	90	357	2576	2898
Maximum	465	439	1182	8884	9823
Plants sampled (#)	207	246	246	246	246

A taxane production index, calculated by multiplying growth rate by the total taxane concentration, was created to estimate the taxane production capacity of individual plants. In our collection, the range in potential taxane production by individual yew plants is greater than 800 fold (Table 10). The yew plant with the highest taxane production index and the mean taxane production index of the top six plants are about 4.7 and 3.7 times that of the average taxane production index, respectively. The growth rate and total taxane rankings of these plants suggest that growth rate and taxane production capacity are about equally important for determining taxane production potential. For example, for the plants with the highest taxane production indices the most important factor is growth rate in two plants, total taxanes in two plants, and nearly equal contributions from growth rate and total taxanes in two plants. The plant with the lowest taxane production index had both growth rate and total taxane content rank at or near the minimum of the 237 plants assessed.

Table 10. *Taxane production index for select yew plants, calculated using 2006 taxane concentrations and 2007 new growth of individual plants, as well as their overall ranking (among 237 plants) for growth and total taxanes (1 = best; 237 = worst).*

Sample	Taxane Production Index	Ranking based on 2007 shoot growth	Ranking based on total taxane concentration
Minimum	3.4	237	219
Average	577.2	n/a*	n/a
Maximum			
Plant #1	2739.2	40	1
Plant #2	2498.4	1	49
Plant #3	1961.8	30	4
Plant #4	1921.3	2	43
Plant #5	1908.1	13	14
Plant #6	1852.1	11	20

*n/a – data not available

In the 2004 CFS plantation, total taxane concentrations were somewhat affected by treatments (Figure 5). Specifically, plants that were mulched had higher total taxanes than those in the control or fertilizer treatments and those treated with compost had more taxanes than those that were fertilized. Most of these differences appear to be due to much higher levels of the taxane 10-DAB in the mulch plus fertilizer and compost treatments (Figure 6). Treatments did not appear to cause any significant differences in concentrations of paclitaxel or DHB (Figure 7).

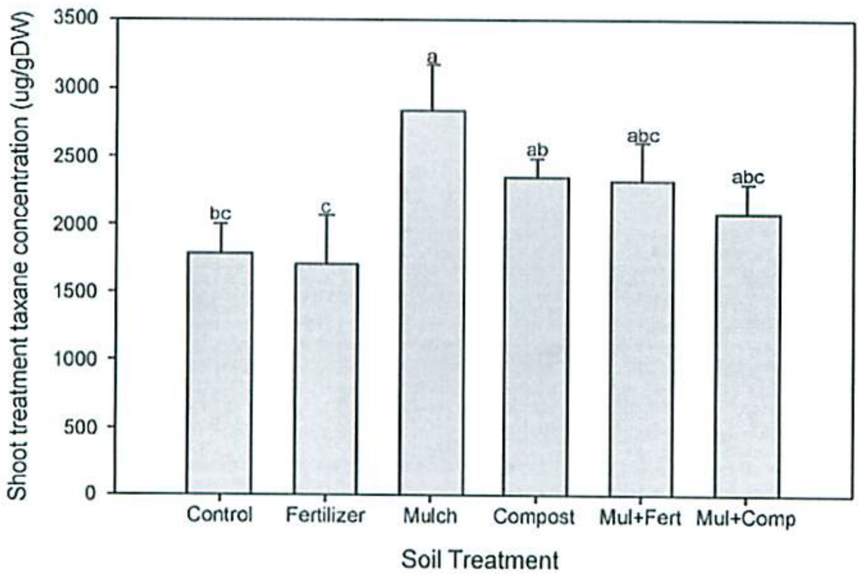

Figure 5. Effects of soil treatments on total taxane concentrations in yew shoots from the 2004 CFS plantation. Error bars = 1 standard error; bars topped by a different letter are significantly different (p ≤0.05).

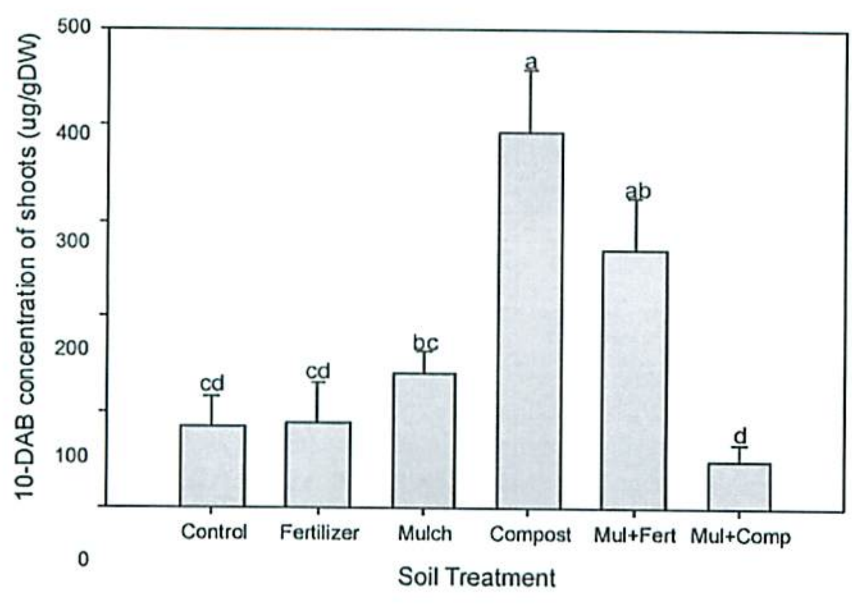

Figure 6. Effects of soil treatments on concentrations of 10-deacetylbaccatin III (10-DAB) in yew foliage from the 2004 CFS plantation. Error bars = 1 standard error; bars topped by a different letter are significantly different (p ≤0.05).

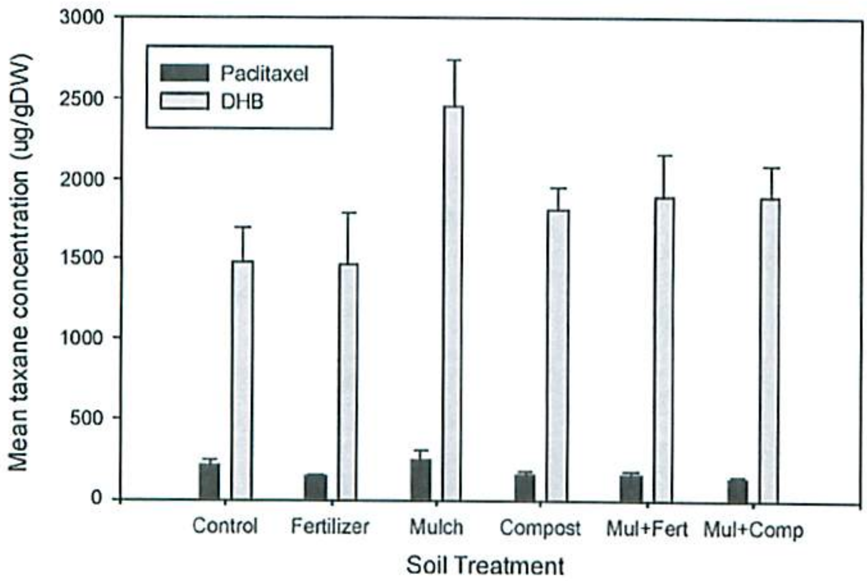

Figure 7. *Effect of soil treatments on paclitaxel and DHB concentrations in yew shoots from the 2004 CFS plantation. Error bars = 1 standard error; no significant differences were found between treatments within taxanes.*

Discussion

In the 2004 CFS plantation, soil treatments significantly affected plant survival. Yew plants in the two compost treatments and the mulch-plus-fertilizer treatments had the highest survival rates, with survival of plants in the single mulch and fertilizer treatments about 10% lower and that for plants in the control plots even lower. Before the fence was installed, observations of plant damage indicated that deer browsing was most severe on the south end of the plantation where the control plots were located, which may have reduced their survival. Survival may also have been influenced by the shoot-to-root balance as the plants grown with the mulch-plus-fertilizer and mulch-plus-compost treatments had significantly better survival rates and lower shoot-to-root ratios (Table 4).

The major treatments influencing growth were the mulch-plus-compost and fertilizer treatments. The mulch-plus-compost treatment produced the most growth, primarily by increasing root growth more than other treatments (Table 4), in part by providing more surface soil moisture for yew's shallow and fibrous root system as well as protection from competition. The major drawback to mulch-plus-compost is its high cost: bulk compost cost about CDN$65 per m^3 and the pine bark mulch was CDN$30 per m^3. At these prices, treating a 1 ha yew plantation with both mulch and compost would cost about CDN$10,000. Fertilizer, the second best treatment for stimulating yew growth, was less than 5% of that cost and should be considered for establishing commercial plantations. Ornamental *Taxus* and tree seedling growth has been shown to be enhanced by mulch with a low (≤25) C:N ratio (Lloyd 2001, Dostalek et al. 2007). *Taxus* grown with high C:N ratio (≥80) wood mulch (similar to the pine bark used in this study) plus fertilizer also increased growth and limited competition from weeds (Lloyd 2001).

In this study, proportional yew growth was calculated using fourth-year new growth divided by third-year total plant biomass to provide an indication of growth rate. Fourth-year CFS plantation proportional growth rate results show that all mulch-treated plants had dramatically increased new growth compared to the other treatments (Figure 4). This growth rate increase may be due to a mulch-related increase in root mass per plant and therefore better balanced plants with lower shoot-to-root ratios. Since mulched soil generally has higher moisture levels in the upper layers than unmulched soil, this growth response may be related to the yew's sensitivity to dry soil because of its shallow, fibrous root system and its preference for moist well-drained sites with high overstory shade (Curtis 1959).

The effects of the soil treatments on growth were expressed in different parts of the plants, which may have implications for plantation culture. For example, yew in the fertilizer treatment had the highest shoot-to-ratio because shoot growth was increased (Table 4). In contrast, yew in mulch treatments, especially when combined with fertilizer or compost, had lower shoot-to-root ratios because root growth was preferentially increased. Since roots are more difficult to harvest completely than shoots, a fertilizer treatment that maximizes shoot growth would be a good choice for a plantation established for a one time whole plant harvest. In contrast, a plantation established with the intent of successive multiple harvests would probably benefit from the growth rate increase (Figure 4) derived from more balanced plants as would be produced by a mulch-plus-fertilizer treatment.

Plant spacing did not affect yew growth in the CFS plantation. Preliminary results from similar CFS yew plantations in New Brunswick and Prince Edward Island also showed no effect of spacing on yew growth (Smith et al. 2006). However, these trials were sampled after only two years of field growth and spacing effects are typically only expressed after a few years when plants start to compete for resources.

One of the critical issues in establishing and maintaining yew plantations is vegetation control. However, actively growing Canada yew plants have been found to be sensitive to direct herbicide spray (Smith, CFS, pers. comm., 2004) so we tried pre-emergence herbicides sprayed on dormant yew plants. Although the Princep® and Goal® had only minor effects on the yew growth and did limit weed growth, they were not particularly effective at controlling all species of weeds. Based on our observation of weed growth, the most efficient means of competition control in this study was a thick layer of mulch combined with some spot applications of glyphosate and, in the individual yew plantation, a layer of landscape cloth covered by mulch. However, the mulch needed spot replacement after 2 growing seasons and the landscape cloth plus mulch was fairly expensive. The next best competition control method was spraying glyphosate during the growing season with the yew plants covered. However, this treatment required repeated applications of at least 3 times during the growing season and was therefore quite labour intensive. In a commercial plantation more automated spray equipment such as a boom or specially designed under-plant sprayer might help to minimize labour costs.

A recent publication by our collaborators (Cameron and Smith 2008) on seasonal variation in taxane concentration of wild Canada yew showed peak concentrations in August and November. A dip in concentration of taxanes occurred between April and August with the minimum occurring in June during active growth. However, between August and April taxane concentrations did not change significantly. Other than avoiding harvest during the summer, seasonal variations in taxane concentrations are unlikely to affect the taxane yields of harvested yew biomass significantly. It is worth noting that the 10-DAB:DHB:paclitaxel ratios reported by Cameron and Smith (2008) were roughly 1:3:1, which are quite different than our approximately 4:20:1 ratios. Our analytical methods were similar to theirs, which suggests northern Ontario clones may have a different taxane distribution than those from eastern Canada. It is also possible that young yew plants in active growth may have different taxane ratios than mature plants growing under natural conditions. Our taxane ratios are closer to those of Zamir et al. (1995) who had DHB:paclitaxel ratios in the range of 5 to 7:1.

The selection of individual plants chosen for fast growth rates and higher taxane production is in its infancy. A large range in growth rates and taxane concentrations was evident in the individual plants (Tables 9 and 10). Based on the taxane production index, the top 6 'elite' individual plants produced taxanes at a rate of almost 4 times the average plant (Table 10). Propagation of these plants for possible plantation establishment was begun in the fall of 2008. However, in the last two years competition from generic drug pharmaceutical companies (especially from India and China) has driven the price of purified paclitaxel from about US $200 down to about US $100 per gram or less. This makes the enhancement of total taxane production on a per hectare basis and semi-synthesis of the 10-DAB and DHB into paclitaxel or docetaxel critical to ensure the economic viability of Canada yew plantations in Ontario.

Using the best growth rate obtained in the CFS plantation, assuming increases in plant survival (to 75%) and growth rate (by 75%) by excluding deer, and with the current price of $6 per kg dry weight of yew biomass, the estimated revenue per ha after 4 years would be about $15,600 (2600 kg dry biomass per ha). The estimated costs would be about $60,000 per ha, which includes the cost of plants, mulch, fertilizer, deer fencing, drip irrigation system, and vegetation management. Estimating revenue using elite cultivars with twice the growth rate and taxane concentrations and a value of $10 per kg of yew biomass (due to taxane concentration doubling), the estimated revenue would be about $52,000 per ha (5200 kg biomass), which is still a loss. By far the largest cost of establishing a yew plantation is that of propagation, estimated at $0.60 per plant with a fully planted ha requiring about 73,000 plants for a total cost of about $44,000. Further research and development to improve propagation methods (Holloway et al. 2008), field crop growth rates, and taxane production capacity of Canada yew will be required to reach the economic threshold of profitability for commercial yew plantation culture in Ontario.

Conclusions

Canada yew can be efficiently propagated and successfully grown in plantations in northern Ontario on a variety of soil types. Canada yew growth can be improved by soil treatments over time, especially fertilization and mulch-plus-compost. Challenges of plantation culture of Canada yew include ensuring affordable weed and, where necessary, deer control. Optimum harvest time is from late summer into fall when taxane concentrations peak (Cameron and Smith 2008). Selection of elite individual yew plants with faster growth and high taxane production was successful. Combining these elite cultivars with improvements in yew propagation will be required to establish profitable yew plantations in Ontario.

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Appendix 1. Growth results from Canada yew plantation culture study

Introduction

The unforeseen complication of deer browsing interfered with our assessment of the effects of soil treatments, soil type, and plant spacing on survival and growth at all three plantation locations. No attempt was made to measure the effect of deer browsing for two reasons. First, this activity was not planned (or budgeted) for and second, due to the random nature of the severity and timing of browsing both within and among locations, it would have been difficult to accurately quantify the degree of browsing and its effects on growth and survival. The most effective deer deterrent (no damage noted for 2 years) was a 2.5-m-high electric fence which, because of its high cost, was installed only at the OFRI arboretum site. The lathe shade covers used at the Thessalon BioCentre were relatively effective (some damage noted around the edges) but only during late fall through spring when the covers were in place. The dog deterrent used at the Whelan farm seemed especially effective in the part of the plantation nearest the farm house. However, the further the plants were from the farm house, the more deer-related damage and destruction of plants that occurred. Irrigation frequency also differed among the three plantation sites with the only the OFRI plantation receiving consistent irrigation at establishment and during both growing seasons. Therefore, all results presented here should be interpreted with these limitations in mind. No discussion of these results is presented because of the difficulties in analyzing results of variables with different degrees of control at different sites.

Results

Plantation Culture Study

Rooting efficiency is a key factor in determining rooting ability of different sources of yew cuttings and gauging the effectiveness of the rooting procedure. Rooting efficiencies of yew cuttings propagated by Thessalon BioCentre averaged over 86%, exceeding 80% for all locations except Dunlop (Table A-1). Although average pre-plant paclitaxel concentrations varied by collection location, these differences were not statistically significant (Table A-1) due to large sample to sample variation in paclitaxel concentrations.

Table A-1. *Average pre-plant current-year shoot paclitaxel concentration and propagation efficiencies of bulk yew collections propagated at the Thessalon BioCentre. Within a column, means followed by the same letter are not significantly different ($p \leq 0.05$).*

Collection location	Number of cuttings rooted	Pre-plant paclitaxel (µg/g DW)	Rooting efficiency (%)
Dunlop	2843	110 a	72.2
Dubourne Lake	1843	n/a	80.9
Basswood Lake	3856	99 a	82.8
Serpent River	4777	179 a	86.6
Boychuck	5242	70 a	92.5
Cranberry	6405	170 a	93.7
Total	24,966	126	86.4

n/a – data not available.

Total survival was greater at the loam site (OFRI arboretum) than the other two sites (Table A-2). However, differential deer browsing was likely the primary reason for the differences in survival since only the loam site had effective year-round deer exclusion. In the three plantations established in 2005, treatments did not significantly affect survival.

Table A-2. *Final survival of yew plants in plantations by treatment and location. Treatment means followed by the same letter are not significantly different (p ≤0.05) (within a location column (excluding total row) and across site totals).*

Treatment	Yew survival (%)		
	Clay site	Sandy site	Loam site
Control	33.9 a	25.0 a	57.9 a
Mulch	34.6 a	25.1 a	45.2 a
Fertilizer	29.9 a	22.0 a	49.5 a
Mulch+Fertilizer	20.6 a	27.4 a	55.9 a
Total per site	30a	25a	51b

n/a = not applicable

At the clay site, yew growth was not affected by the treatments and at the sandy site was only marginally affected (Tables A-3 and A-4). Plant spacing did not affect growth (data not shown). No differences in the amount of new shoot growth were observed at the clay site or in the root or total plant biomass at the sandy site. At the sandy site, shoot growth of plants in the control treatments was higher than that of plants in the mulched treatments. Small differences in shoot-to-root ratio were observed at the BioCentre plantation. However, these differences are likely confounded by deer browsing effects.

Table A-3. *Effect of soil treatments on yew shoot growth (grams dry weight) at the clay site. Means followed by the same letter are not significantly different (p ≤0.05).*

Treatment	new shoot growth per plant (g DW)
Control	0.94 a
Mulch	0.94 a
Fertilzer	0.92 a
Mulch+Fertilizer	0.98 a

Table A-4. *Effect of soil treatments on biomass (grams dry weight) and shoot:root ratios of yew plants harvested in 2007 from the sandy site. Within a column, means followed by the same letter are not significantly different (p ≤0.05).*

Treatment	Biomass (g DW) per plant			Shoot:root ratio
	shoot	root	total	
Control	0.91 a	0.86 a	1.77 a	1.07 a
Mulch	0.69 b	0.87 a	1.56 a	0.80 c
Fertilzer	0.86 ab	0.90 a	1.75 a	0.94 ab
Mulch+Fertilizer	0.77 ab	0.89 a	1.66 a	0.87 bc

In the loam plantation, after two growing seasons treatments had minimal effects on the growth of yew plants (Table A-5). The mulch-plus-fertilizer treatment stimulated more root growth and plants in that treatment had a significantly lower shoot-to-root ratio than did fertilized yew plants. No significant effects on shoot growth or total plant growth were found.

Table A-5. *Effect of fertilizer treatment on total biomass (grams dry weight) and shoot:root ratios of yew plants harvested in 2007 from the loam site. Within a column, means (n=721 plants) followed by the same letter are not significantly different (p ≤0.05).*

Treatment	Biomass (g DW) per plant			Shoot:root ratio
	shoot	root	total	
Control	2.48 a	1.16 ab	3.64 a	2.17 ab
Fertilizer	2.08 a	0.92 b	3.00 a	2.26 a
Mulch	2.06 a	1.04 ab	3.10 a	2.01 bc
Mulch+Fertilizer	2.57 a	1.43 a	4.00 a	1.85 c