PROPAGATION OF *Camptotheca acuminata*

A Thesis

by

DOUGLAS WAYNE MAXWELL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2003

Major Subject: Horticulture

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Approved as to style and content by:

R. Daniel Lineberger (Chair of Committee)	Fred T. Davies (Member)
W. Todd Watson (Member)	Tim Davis (Head of Department)

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ABSTRACT

Propagation of *Camptotheca acuminata*.

(May 2003)

Douglas Wayne Maxwell, B.S., Texas A&M University

Chair of Advisory Committee: Dr. R. Daniel Lineberger

Research was undertaken to optimize propagation of the southern China native *Camptotheca acuminata* Decaisne, source of the medicinal compound camptothecin that is used in the treatment of multiple forms of cancer and other diseases. The study focused on cutting propagation, micropropagation, and seed storage.

Softwood cuttings of *C. acuminata* rooted readily in intermittent mist (4 sec on every 6 min.) in coarse vermiculite when treated with K-IBA (indolebutyric acid, potassium salt) quick dips ranging from 4.14 mM to 37.3 mM, with a 29.0 mM quick dip (5 sec.) promoting 82% rooting with little foliar damage. Actively growing shoot tip explants were tissue cultured on media containing Murashige and Skoog, Gamborg's B5, and Woody Plant Medium (WPM) salts in factorial combinations with BA (benzyladenine). WPM containing 4.44 μM BA promoted excellent shoot proliferation; microcuttings were rooted, acclimated, and grown in the greenhouse. Seeds stored in polyethylene bags in a refrigerator (4°C) or freezer (-20°C) maintained good germination (81% and 80%, respectively) while seeds stored at room temperature (25°C) in polyethylene bags lost germination ability quickly (58%) after one year of storage. *C.*

acuminata is readily adaptable to modern nursery techniques for either vegetative or seed propagation.

DEDICATION

This thesis and this research are dedicated to Miss Zora Lane. Her passion for gardening inspired my interest and love of horticulture. Her fight with cancer is evidence of the need for research in this field.

ACKNOWLEDGMENTS

I would like to thank Dr. Dan Lineberger for his continued guidance in all areas of my professional career. He has played an important role in the majority of my academic and professional successes over the course of my time at Texas A&M University, as I am sure he will continue to do. I am grateful to Dr. Fred Davies, Dr. Roberta Smith, and Dr. Todd Watson for their guidance with my research and serving on my committee. Partial funding for this research was provided by the Xylomed Foundation. Organizations such as this make continued research in this field possible. I also thank Dr. Jerry Parsons and Gretchen Chaffin for their skills in harvesting *C. acuminata* seed. I am forever indebted to my family for not questioning my dreams and always standing behind me. Finally, I greatly appreciate and thank my extended family in the Horticulture / Forest Science Building. My time on this campus would not have been the same without this truly genuine group.

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INTRODUCTION

Background

When one ponders the most destructive and yet incurable diseases of our time, cancer and AIDS are definitely top of the list. Since 1990, approximately 11 million new cancer cases have been diagnosed in the United States. This year, about 564,800 Americans are expected to die of cancer, over 1,500 each day (American Cancer Society, 2002). The number of HIV infections in the US is estimated at 850,000 to 950,000. At least 40,000 new HIV infections are occurring each year in the United States (National Institute for Allergies and Infectious Diseases, 2002).

Vast human resources have been invested in developing effective treatments and cures. Medical researchers have long since looked toward plants for answers in these races for life and understanding (Cseke and Kaufman, 1998). Botanical reserves have yielded such medicinal compounds as Taxol (*Taxus brevifolia*) (Monroe and Mansukh, 1996), catharanine, vincristine and vinblastine (*Catharanthus roseus*) (Snyder, 1996) and delta 9 and 11 THC (*Cannabis sativa*) (National Commission on Marijuana and Drug Abuse, 1972). One of the latest additions to this growing list is camptothecin (CPT). This compound and conjugates thereof have shown evidence of fighting both cancer and AIDS (Monroe and Mansukh, 1996; Pantazis, 1996; Priel, Showalter, and Blair, 1991; Research Triangle Institute, 1997).

This thesis follows the style of the Journal of The American Society for Horticultural Science.

Camptothecin

Camptothecin is a monoterpene-derived indole alkaloid produced by *Camptotheca acuminata* Decaisne, a tree in the Nyssaceae family (Vincent et al., 1997). The compound is found in most parts of the tree including roots, wood, fruit, bark, and leaves. CPT was first isolated at the Research Triangle Institute in the 1960's (Wall, Wani and Cook, 1966). Clinical trials began in the 1970's, but exhibited such high toxicity levels that interest was lost in the compound. In 1985, Haissig discovered that CPT acted in the inhibition of DNA replication (McDonald, 1997); camptothecin research again gained interest. Later efforts have been more successful resulting in a number of drugs currently in use (Creemers, et al, 1996; Dancey and Eisenhauer, 1996; Takimoto, Wright and Arbuck, 1998).

Anti-Cancer Properties

The anticancer properties of CPT were first discovered in 1958 by Dr. Monroe E. Wall, USDA and Jonathon Hartwell, National Cancer Institute (McDonald, 1997). The anticancer activity of this compound and its conjugates is due to the inhibition of DNA topoisomerase I, inhibiting DNA replication in infected cells (Kjeldsen, Svejstrup and Gromova, 1992). Camptothecin is currently used as a chemical precursor for the semisynthetic derivatives topotecan and irinotecan. These drugs received FDA approval for human application and testing in 1996. Other water-soluble derivatives are being tested and in line for approval, including drugs for ovarian, colorectal, breast, pancreatic, and other cancers (Heron, 1998; McDonald, 1997).

Anti-Retroviral Properties

The CPT extract has been shown also to inhibit retroviruses (Priel, Showalter and Blair, 1991). These inhibitory effects have been shown currently against HIV and Equine Infectious Anemia Virus. The activity is due to inhibition of TAT-mediated transcription from the viral promoter (Li, Wang and Pardee, 1994).

Camptotheca acuminata

C. acuminata, also called Tree of Joy or Xi Shu, originates from areas of Southern China and Tibet. The tree was first introduced in the United States by E.H. Wilson in 1911. Since then, it has been grown in botanical gardens, arboreta, and small plantations around the country (McDonald, 1997). The species prefers warm, humid, subtropical climates; therefore, cultivation efforts have focused on the southeastern US. Known plantings are found in Arkansas, California, Hawaii, New Jersey, South Carolina, Texas, and Louisiana. The Xylomed Research Consortium, a non-profit group aimed at promoting research and development of CPT, has risen as a major player in this process by establishing a number of these plantations.

While CPT seems to be a viable precursor to some groundbreaking new drugs, production of the compound is not without challenges. The availability and cost of raw CPT in the US are severe limitations to further implementation. The tree was proposed in 2000, although withdrawn, for protection by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2000). The use of CPT in China is limited due to its value of export with estimated cost per kilogram of $35,000 and the commercial synthesis of CPT is not considered a viable option (Li and Adair, 1994).

This leaves the cultivation of *C. acuminata* and efficient production of CPT within the US a prime priority.

A vast array of research institutions are currently at work developing enhanced techniques for the culture and sustained harvest of this crop, investigating the molecular mechanism behind the production of CPT, creating more valuable clones of the tree, and studying means of propagation of the species. The latter is the goal of this lab. A series of experiments have been performed to investigate vegetative propagation as well as seed storage of the tree.

Research Goals

1) Develop a practical *ex vitro* propagation scheme for *Camptotheca acuminata*.
2) Develop a practical *in vitro* propagation scheme for *Camptotheca acuminata*.
3) Describe effective methods for long term storage of seed.

LITERATURE REVIEW

Previous Research

Cutting Propagation

Propagation techniques using cuttings have been an effective means of multiplying a variety of tree and other woody species (Dirr, 1990). Terminal semi-hardwood and hardwood shoot cuttings have been commonly chosen explants for propagation by cuttings (Southworth and Dirr, 1996; Hinesley and Snelling, 1997; Meahl and Lanphear, 1967; Khattak et al., 1999). Reduction in leaf surface area of these cuttings has proven to additionally enhance propagation success in many woody species including tropical trees (Brennan and Mudge, 1998.; Teklehaimonot et al., 2000).

Application of a variety of growth regulators has been shown to promote rooting success of woody plant cuttings. Of these thousands of chemicals tested over the years, Indole-3-butyric acid (IBA) and Napthalenacetic acid (NAA) are used with IBA being the most commonly used plant growth regulator for a variety of rooting promoting treatments (Dirr and Heuser, 1987; Haissig, 1979; Haissig, 1983).

IBA and the potassium salt of IBA (K-IBA) applied with a “quick dip” treatment to stem cuttings have been used to successfully and efficiently propagate woody ornamental and tree species (Still and Zanon, 1991; Al-Saqri and Alderson, 1996). Dirr (1990) used 0.5% IBA in a 95% ethanol solvent to apply a 5 second “quick dip” before successfully rooting a variety of woody landscape ornamentals in a peat:perlite medium. Southworth and Dirr (1996) found the highest rooting success with 10,000 mg/l K-IBA

treatments on stem cuttings of *Cephalotaxus harringtonia*, a medicinally important tree species. As well, there was an indication that K-IBA was less injurious to cuttings than the free acid of IBA, noting that IBA produced basal necrosis at the concentrations tested. Tropical trees such as *Parkia biglobosa* and *Inga feuillei* have also been rooted successfully with the aid of IBA "quick dips" (Teklehaimonot et al., 2000; Brennan and Mudge, 1998).

K-IBA and ethanol treatments have also produced high rooting success in *Photinia* x *fraseri* where Dirr (1989) found that the ethanol carrier also aided in root promotion; although, ethanol alone did not produce rooting success. Similar results with *Betula platyphylla* var. *szechuanica* indicated an ethanol carrier influence (Kling et al., 1985).

Following the "quick dip" application of auxin treatments, the environment can also play a role in successful rooting of tropical tree species by cuttage. Intermittent mist application to reduce respiration of the explants has shown beneficial in promoting the formation of adventitious roots (Brennan and Mudge, 1998). Other research has shown no significant advantage of mist application to tropical tree species indicating that these leafy explants may photosynthesize during propagation (Newton and Jones, 1993).

Micropropagation

Propagation of plant species *in vitro* through the use of tissue culture techniques requires the establishment of an appropriate explant type for initiation of the culture, growth regulators and medium for multiplication of the species as well as a scheme for rooting and acclimatizing the new plantlets to *ex vitro* growing conditions (Murashige,

1974). Plants are micropropagated for a variety of reasons including difficulty propagating by traditional vegetative propagation schemes (Lloyd and McCown, 1980), poor seed germination (Pereira-Netto, 1996), and the production of phenotypically uniform plants (Lineberger, 1983).

While the basic principles of tissue culture techniques are the same for all plant species, the culture of trees is often distinguished from that of other species. This is due to the heterogeneous nature of tree populations, which leads to a wide range in responsiveness to culture treatments, and due to a lessened responsiveness of mature woody tissues to culture treatments as compared to juvenile tissues (Sommer and Caldas, 1981). Despite this, a variety of tree species have been successfully micropropagated from mature explants (James et al., 1988; Druart, 1980; Swartz et al., 1990; Leblay et al., 1991; Chevreau et al., 1989; Gupta et al, 1980; Rao et al., 1998).

A variety of explants have been targeted as tissue sources for successful micropropagation. Studies aimed at enhancing shoot multiplication by lateral bud breaks focus on excised buds and shoot tips (Wiskson and Thimann, 1958; Hasegawa, 1980) while adventitious shoot formation can occur with leaf tissue explants (Leblay et al., 1991; Chevreau et al., 1989). In addition, juvenile tissues are often targeted for micropropagation studies prior to development of a scheme for multiplication of mature tissues (Gupta et al, 1980). Jain and Nessler (1996) successfully multiplied *C. acuminata in vitro* utilizing 30 day old seedling shoot tips. The explant chosen and the mechanism by which the species will produce multiple shoots will affect the need for

nutrient combinations and plant growth regulators in the tissue culture medium to aid the formation of shoots.

Determination of the most advantageous tissue culture medium is often conducted by screening a variety of commonly used media, producing varying results. Brand and Lineberger (1986) found a Murashige and Skoog (MS) medium unsatisfactory in the propagation of *Halesia* as compared to Woody Plant Medium (WPM). Venketeswaran et al. found no significant response between MS and WPM media with micropropagation of Mahogany. Jain and Nessler (1996) found Gamborg's B5 medium most advantageous with *C. acuminata* seedling explants.

Plant growth regulator treatments applied in the culture medium can significantly enhance the multiplication rates of tissue culture systems. Leaf explants have been shown to produce higher rates of adventitious shoots in tree species with a combination of auxin and cytokinin growth regulators (Leblay et al. 1991; Chevreau et al., 1989). Shoot tip explants have been most influenced by cytokinin treatments due to the antagonism of auxins and cytokinins and their affect on apical dominance (Wiskson and Thimann, 1958; Coenen and Lomax, 1997). Of cytokinins used in micropropagation, N_6-benzyladenine (BA) is the most commonly used in many species as a principle or the sole growth regulator (Lineberger, 1983; Hasegawa, 1980; Heloir et al., 1997; Jain and Nessler, 1996; Liew and Teo, 1998; Ajithkumar and Seeni, 1998). This includes a variety of tropical and medicinal trees.

Upon the successful multiplication of a species *in vitro* a scheme for the rooting and acclimatization of the plantlet is required. Evidence has been found in the literature

to support *in vitro* and *ex vitro* rooting methods. Ajithkumar and Seeni (1998) found a one-half strength MS medium with 0.5 and 10.0 mg/l IBA to be the optimum rooting medium for the medicinal tree *Aegle marmelos*. Other research supports that one-half and one-fourth strength MS salt formulations with auxins promote *in vitro* rooting and that increasing the salt concentration of the medium and adding a cytokinin both negatively affected rooting response (Hasegawa, 1980; Pereira-Netto, 1996). Other researchers have indicated little response to auxins *in vitro* (Teklehaimonot, 2000) and *ex vitro* (non-sterile) rooting environments equally or more advantageous to rooting response (Brand and Lineberger, 1986; Cummins and Asby, 1969).

The acclimatization of the newly rooted plantlets to standard growing conditions can be vital in the success of the micropropagation program (Sommer and Caldas, 1981). Gradually decreasing relative humidity through the use of humidity tents or other forms of intermediate humidity environments has been evidenced to increase plantlet survival rate in the micropropagation of tropical trees (Pereira-Netto, 1996; Rao et al., 1998).

Seed Storage

The prolonged storage of seed has been an important consideration and point of research for many forest trees. Progressive forest decline has prompted conservation strategies to prevent loss of genetic diversity (Pita et al., 1998). Seed banking has been considered the most efficient method of preservation for most tree species. However, the rapid decrease of seed viability evident in many of these species requires controlled environment storage regimes to prolong the viability of stored seed.

Cold storage has proven to be an effective means of maintaining seed germination percentages in a variety of species (Carpenter and Boucher, 1991; Clark and Moore, 1993). Species of pine have been successfully stored at low temperatures (4°C) for 50 years while remaining viable, although at reduced germination percentages (Barnett and Vozzo, 1985).

Similar storage techniques have been applied to tropical and subtropical tree species. Gonzalea and Fisher (1997) found storage temperatures of 4°C ± 1°C allowed for seed storage in excess of 6 months with only moderate reduction in germination rates in three tropical tree species. Teak seed were stored for 10 years at 4°C while maintaining appreciable germination rates (Verapong-Suangtho, 1992). Storage environments range from open containers to partially and completely sealed vessels for the duration of the treatments. Verapong-Suangtho (1992) also reported that no significant difference was found between sealed and non-sealed containers.

Perdue and Smith (1970) tested storage of *C. acuminata* fruit in moist sand sealed in polyethylene bags. The specimens were stored 30 days at 4.4°C and room temperature. They observed slightly higher germination percentages with the cold-treated indicating that the species could benefit from stratification. They also reported that dry seed produced higher germination percentages. The result was an informal hypothesis that the best germination percentages would be produced with dry stratification of seed.

MATERIALS AND METHODS

Very little reliable information is available at this point on the propagation of *C. acuminata*. Therefore, a set of experiments was outlined to develop practical means of propagating this species. Experiment one was set up to test the feasibility of using cutting propagation to multiply the species. Experiment two used tissue culture methods to clonally propagate the plant *in vitro*. Finally, experiment three approached the topic using sexual reproduction, studying seed storage effects on seed germination.

Experiment 1 – Propagation by Cuttings

Objective

Asexual propagation using softwood cuttings is the industry standard alternative to seed propagation or micropropagation. Varied success using softwood cuttings of woody plants has been reported (Dirr, 1989; Dirr, 1990). The ability to effectively propagate this species using *ex vitro* means could benefit commercial production facilities that may lack the capability to propagate the species under sterile conditions.

The application of the auxin indole-3-butyric acid potassium salt (K-IBA) has been reported to enhance rooting success in a wide variety of species (Haissig, 1979; Haissig, 1983; Dirr, 1983; Dirr, 1990). This study tested the effects of K-IBA on rooting of *C. acuminata* softwood cuttings.

This set of experiments was designed to develop an *ex vitro* propagation scheme for softwood cuttings of *C. acuminata*. It is hypothesized that the application of IBA

through a quick dip application of K-IBA will increase percent rooting and overall root quality (number of roots) over the control treatment.

Explants

Plant material used for this study was obtained arbitrarily from eight three year old clonally produced trees grown on the campus of Texas A&M University. Five node softwood cuttings, counted basipetally from the apex, served as the explant for the treatments. The cuttings were further prepared by removing leaves from all nodes except two developed nodes near the apex. The experiments were timed during active growth of the trees during early to mid summer (June and July, 1999). Bundles of ten cuttings each served as replicates within the treatments. The experiment was repeated twice in time, sequentially.

Treatments

K-IBA treatments of 4.14, 12.4, 20.7, 29.0, and 37.3 mM were prepared by dissolving reagent grade IBA in reverse osmosis purified water with the dropwise addition of KOH until the acid dissolved (Sigma Chemical Co., St. Louis, MO) (Dirr, 1990; McGuigan et al., 1996). Water alone served as the control treatment. A quick dip application of five seconds was applied to the cuttings at a dipping depth of 2 cm. (Dirr, 1983; Dirr, 1990; Southworth and Dirr, 1996). The cuttings were then planted immediately in horticultural grade vermiculite (previous unpublished trials by Matt Kent, Texas A&M University; Al-Saqri and Alderson, 1996). Cuttings were stuck on 4 cm centers at a depth of 4-5 cm.

A randomized block experimental design was used by randomly assigning six treatments in each of three blocks. The application was made to three bundles per treatment, with a total of 30 cuttings per treatment and 180 cuttings per experiment. The second replicate in time identically reproduced the above treatments.

Following treatment, the flats of cuttings were placed under intermittent mist set at 4 seconds on ever 6 minutes. The greenhouse averaged a daily maximum temperature of approximately 32°C and reached a daily maximum photosynthetic photon flux (PPF) of approximately 900-1000 $\mu Mm^{-2}s^{-1}$ (measurements recorded with a Li-COR quantum sensor, Lincoln, NE, USA).

Experiment 2 – Micropropagation of *Camptotheca acuminata*

Objective

This phase of research was directed toward developing a practical and efficient means of multiplying *C. acuminata* using standard tissue culture methods. Previous research by Jain and Nessler (1996) used shoot tips from 30 day old seedlings and reported best results using a two stage liquid to semi-solid medium of Gamborg's B5 (Gamborg, Miller, and Ojima, 1968) containing 17.4 μM BA (approx. 4 mg/l). Liu and Li (2001) cultured lateral buds of three year old seedlings established on BA free B5 medium then transferred to B5 containing 4.4 μM (approximately 1 mg/l) BA + 0.54 μM NAA (α-napthaleneacetic acid). The goal of this experiment was to improve the practicality of these previously published methods by using two-year-old trees, multiple explant types, a single culture medium, and lower concentrations of plant growth

regulators as appropriate to develop a practical micropropagation establishment scheme for *C. acuminata*.

Two explant sources, newly expanding leaves (Al-Juboory, Skirvin, and Williams, 1998; Arena and Pastur, 1997) and rapidly growing shoots harvested from one-year-old trees, were tested in this experiment on 3 basal media with varying levels of BA. Propagules were later rooted *ex vitro* as well as *in vitro* on an IBA containing B5 medium.

Medium

A 3 x 5 factorial experimental design was used to test three standard plant tissue culture media -- Gamborg's B5 (Gamborg et al., 1968), Murashige and Skoog (Murashige and Skoog, 1962), and Woody Plant Medium (Lloyd and McCown, 1981). One or more of the media tested was expected to meet the nutrient requirements of the explant.

Plant Growth Regulator

Various concentrations of the cytokinin 6-benzyladenine (BA) were added to the media to promote multiplication. BA is the sole plant growth regulator tested based on a cytokinin factorial experiment conducted by Jain and Nessler (1996) as well as preliminary trials in this lab.

The variable combination of medium and BA concentration comprised the treatment variables used for each explant source in this experiment (Table 1). Each medium contained 3% sugar, 0.6% agar (Bacto® agar, Difco), and the pH adjusted to 5.3 for WPM and 5.7 for MS and B5.

Table 1. Factorial treatments of medium, BA concentration, and explant type used in the micropropagation experiment.

	B5	MS	WPM
0 μM BA	Shoot	Shoot	Shoot
	Leaf	Leaf	Leaf
4.44 μM BA	Shoot	Shoot	Shoot
	Leaf	Leaf	Leaf
8.88 μM BA	Shoot	Shoot	Shoot
	Leaf	Leaf	Leaf
17.8 μM BA	Shoot	Shoot	Shoot
	Leaf	Leaf	Leaf
35.5 μM BA	Shoot	Shoot	Shoot
	Leaf	Leaf	Leaf

Explants

Newly expanding leaves and growing shoots were the tested explant types. All explants were obtained from two-year-old trees of a single clonel of *C. acuminata*. The explants were sterilized in 20% sodium hypochlorite (shoot tips) or 15% sodium hypochlorite (leaves) containing a drop of Tween 80 for 20 minutes and subsequently triple rinsed in sterile distilled / deionized water.

Explants were plated on Petri dishes (100 x 15 mm) corresponding to each of the 15 media and growth regulator treatment combinations. The explants were cultured under a 16 hour photoperiod of fluorescent lights with a temperature of approximately 25° C for 8 weeks until harvesting.

Establishment

Microcuttings taken from these cultures were rooted *in vitro* on a medium containing WPM with varying concentrations of indole-3-butyric acid (IBA) to promote root formation *in vitro* (Jain and Nessler, 1996; Liu and Li, 2001). Treatments tested were 0 μM, 0.49 μM, 4.92 μM, and 9.84 μM IBA. Following root formation, rooted cuttings were transferred to humidity domes and established in fine soilless growing mix (Redi-Earth, Scotts-Sierra Inc., Marysville, OH) and allowed to acclimatize in this high humidity environment under fluorescent lights for 3 weeks before moving to greenhouse growing conditions.

Experiment 3 – Seed Storage of *Camptotheca acuminata*

Objective

While clonal propagation of *C. acuminata* is one of the primary objectives of this research, sexual propagation of the species is currently the primary method employed (Li and Adair, 1994). Also, since the tree is native to the eastern hemisphere, the introduction of new germplasm in this hemisphere may face extended storage conditions until planting. To improve germination percentages of this valuable crop, this experiment tested cold storage treatments over two years.

Seed Source

Seed for these experiments was obtained from a single tree at the San Antonio Botanical Garden, the same source as the other material used in this research. By using a single seed source, variation introduced by genetic diversity is controlled.

Storage Treatments

Four storage conditions were tested to determine the optimum storage temperature to maintain the germination percentage of *C. acuminata* seed:

- 22 to 27°C (room temperature)
- 22 to 27°C (room temperature) Unsealed
- 0 to 4 °C (commercial refrigerator)
- -18 to -20 °C (commercial freezer)

The two cold treatments as well as a control were sealed in polyethylene bags (Ziploc® 1 gallon size storage bags, DowsBrands L.P., Indianapolis, Indiana) during storage. To eliminate the effect of the sealed environment, a second room temperature control was tested and compared to that of the sealed control treatment. Temperature readings in the storage environments were taken periodically over time using a laboratory thermometer to determine the actual average temperature range each of the treatments.

Germination

At 6 month intervals, a sample of the stored seed was sown in a soilless medium (Sunshine No. 1; Fison Hort., Vancouver, BC) and germinated under standard greenhouse cultural conditions (10/1998, 4/1999, and 10/1999). In the fall of each year, new crop seed was harvested and entered into the storage experiment to replicate the analysis. This process was conducted over two years.

RESULTS

Experiment 1 – Propagation by Cuttings

Six weeks following cutting, the study was harvested and quantitative data were collected on rooting percentage and root quality, both factors being important when evaluating the viability of a propagation scheme (Dirr, 1983).

Rooting

The K-IBA treatment concentration was determined to have a significant effect on rooting percentage by analysis of variance at $p \leq 0.0001$ (Table 2). Means of rooting percentages were also separated by LSD at $\alpha = 0.01$ showing significant differences between the treatment levels. Rooting percentage data were found to follow a normal distribution using the Shapiro-Wilk and Kolmogorov-Smirnov tests (not normally distrbuted at $p \leq 0.3$).

Table 2. Effect of K-IBA quick dip treatment on rooting of *Camptotheca acuminata* softwood cuttings.

Treatment	% Rooting	Roots/Cutting	Total Root Length/Cutting (cm)
29.0 mM	67.1[z] a[y]	3.87[z] a[y]	31.4[z] a[y]
37.3 mM	58.6 ab	2.07 b	15.7 b
20.7 mM	52.1 b	1.47 bc	9.50 bc
12.4 mM	32.6 c	0.57 cd	3.71 cd
4.14 mM	24.9 cd	0.22 d	1.49 cc
0 mM	9.22 d	0.08 d	0.50 d
Significance[x]	***	***	***

[z] Means for n = 360
[y] Arc-sin transformed mean separation within columns at $\alpha = 0.01$ by LSD
[x] NS, *** Nonsignificant or significant at $p \leq 0.0001$ respectively

Rooting followed a saturation curve leveling off near the upper range of the experiment. The quick dip treatment of 29.0 mM K-IBA for five seconds produced optimal rooting with an average rooting percentage of 81.7% (non-transformed). The standard curve of rooting percentage of replicate averages (n = 360) was found by regression analysis with $R^2 = 0.74$ and indicates optimal rooting success between 29.0 mM and 37.3 mM (Fig. 1).

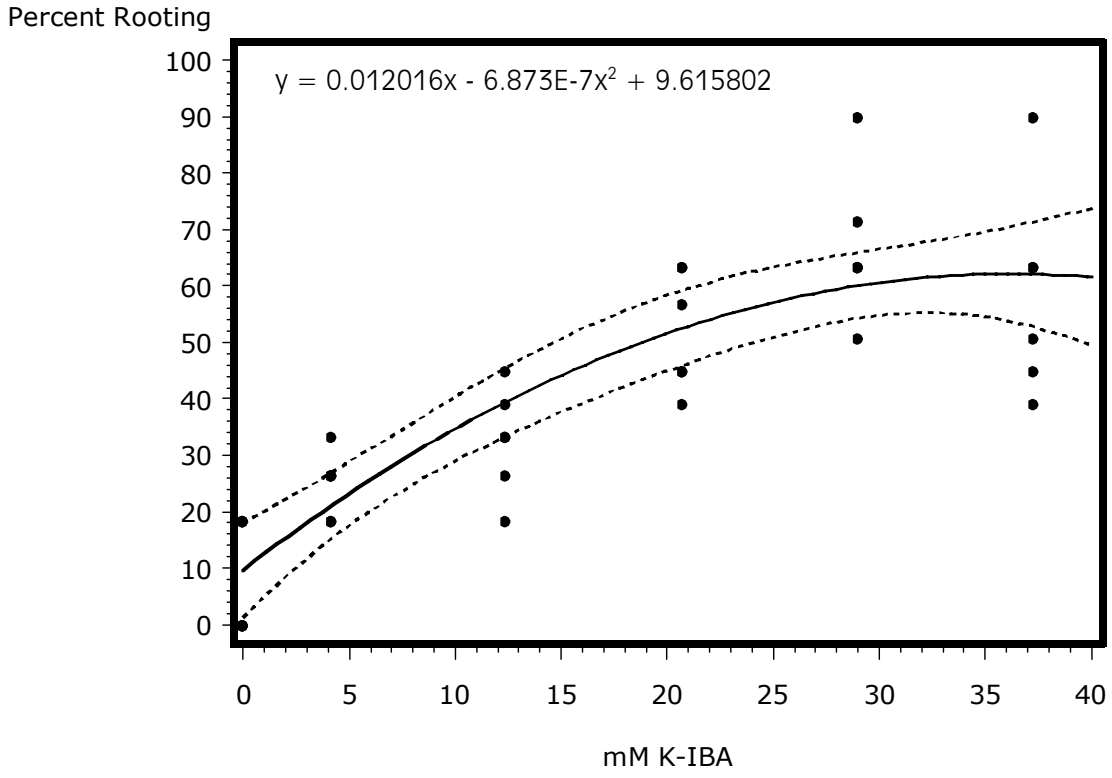

Fig. 1. Regression analysis of percent rooting as affected by K-IBA concentration in 5 second quick dips of softwood cuttings. Combined arc-sin transformed rooting percentage data from replicate trials were analyzed using regression to produce a saturation curve (solid line) and a 95% confidence interval (dotted lines) plotted against percent germination data points.

Root Production

The total number of roots per cutting followed a similar trend over the treatments (Fig. 2). The number of roots per cutting was significantly affected by the K-IBA treatment concentration, determined by analysis of variance at $p \leq 0.0001$ (Table 2). Roots over 2 cm in length at the time of harvest were counted. Cuttings treated with 29.0 mM K-IBA exhibited the highest root production. This treatment averaged 3.87

roots per cutting (n = 360), which was significantly greater ($\alpha = 0.01$) than the 37.3 mM treatment concentration (Table 2).

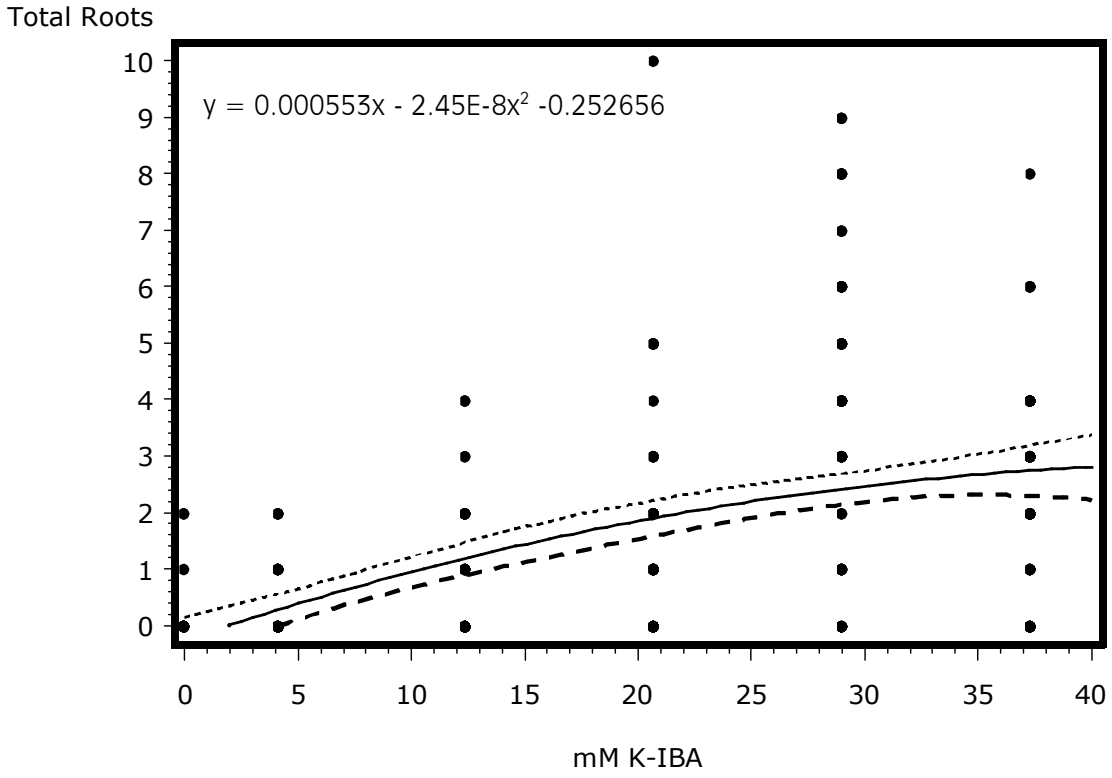

Fig. 2. Increase in average number of roots produced per cutting affected by increasing K-IBA concentration in quick dip treatments.

Root Length

As another measure of root quality, the length of all roots produced per cutting was recorded. Total root length per cutting was significantly affected by the K-IBA quick dip treatment concentration at $p \leq 0.0001$ (Table 2). Regression analysis showed that this measurement follows a trend similar to percentage rooting and number of roots per cutting (Fig. 3).

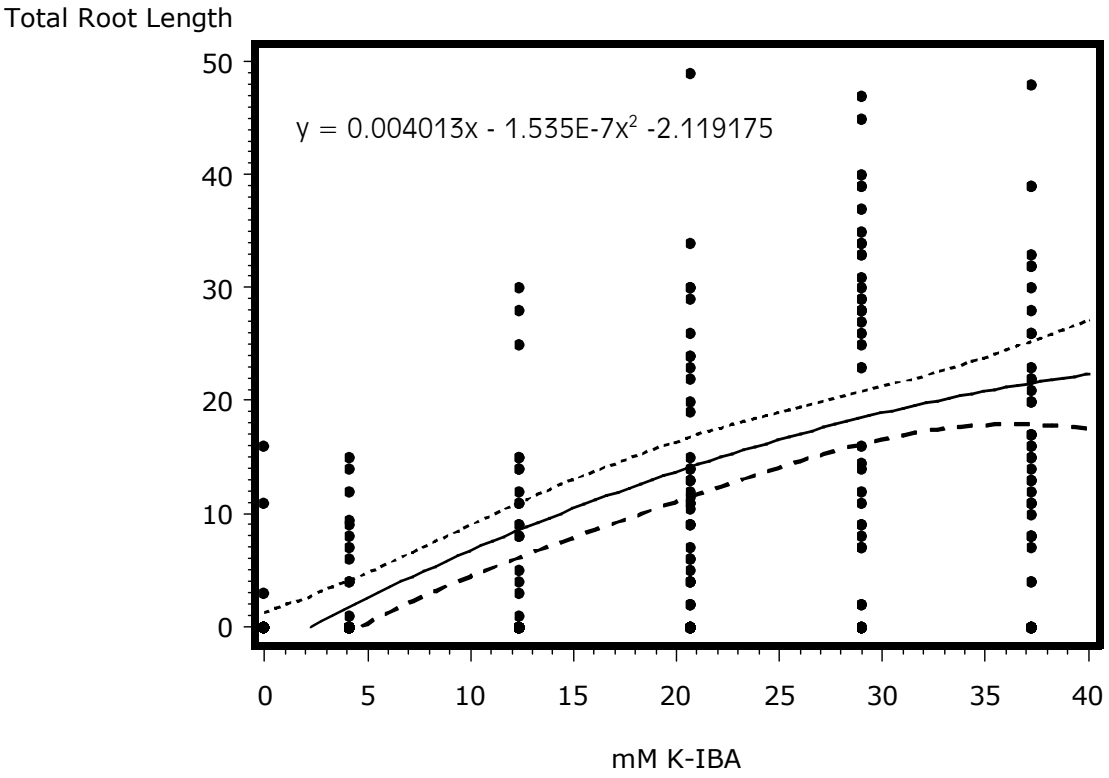

Fig. 3. Total root length per softwood cutting as affected by K-IBA quick dip treatment. Regression curve is plotted against combined replicate data points for each treatment level.

The experiments were photographed at harvest to show the visual difference between each treatment (Fig. 4). Representative cuttings exposed to each of the six treatment levels showed the increasing rooting success with exposure to a K-IBA quick dip. Also noteworthy was the degree of vigor the cuttings displayed. Upon transplanting rooted cuttings to a soilless peatlite mix (Sunshine No. 1; Fison Hort., Vancouver, BC) in a greenhouse, less than 20% of the cuttings exhibited a lag in growth following rooting.

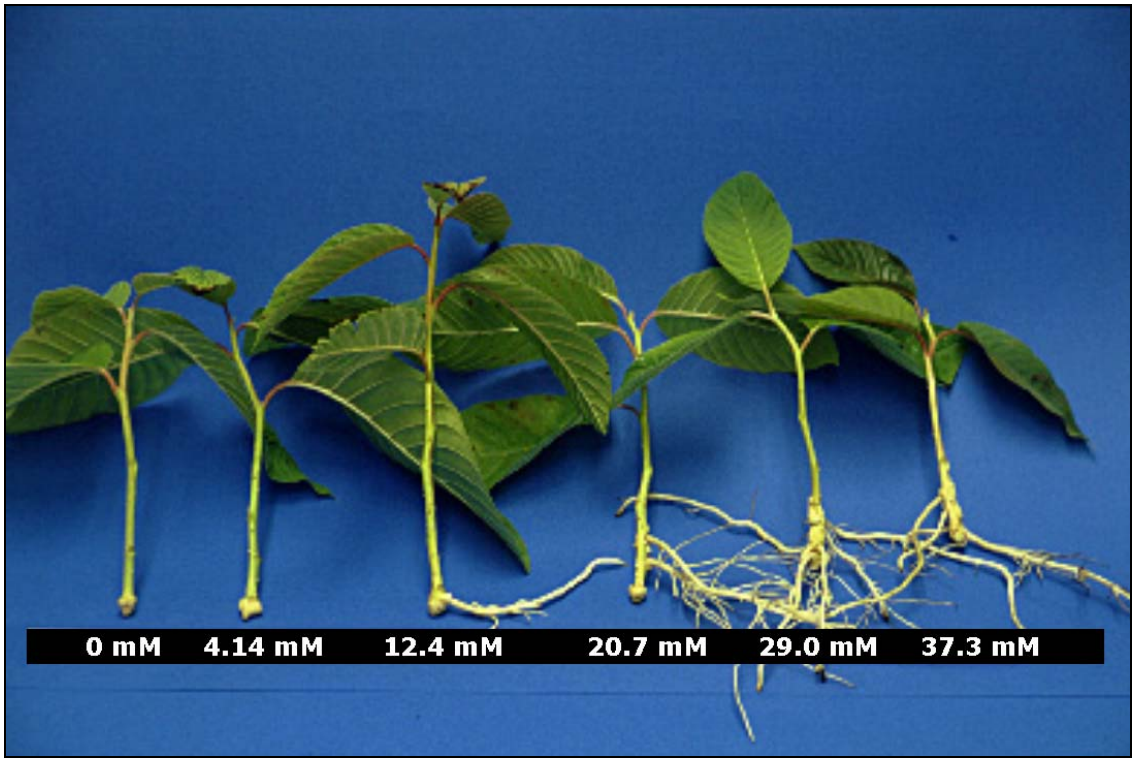

Fig. 4. Photo of representative softwood cuttings treated with one of the K-IBA quick dip treatments. Visual observations taken at time of harvest (6 weeks following cutting) exhibit the effect of K-IBA concentration on root production and total root length per treated cutting.

Conclusion

Rooting success over 50% is often deemed adequate for commercial production of tree species (Goh, et al., 1995; Khattak, et al., 1999). Using this to evaluate the usefulness of the propagation scheme developed for *C. acuminata*, it is concluded that the application of a 5 second quick dip of 29.0 mM K-IBA to softwood cuttings of *C. acuminata* is a viable means of propagation for a commercial setting. Cuttings treated with this application exhibited 81.7% (non-transformed) rooting success and averaged 3.87 roots/cutting. Furthermore, the ease of treatment application as well as the high

rooting percentages observed showed this species could be easily adapted to a commercial production facility, which lacks the requirements for *in vitro* propagation.

Experiment 2 – Micropropagation of *Camptotheca acuminata*

Explants were cultured on the various media treatments for 10 weeks until they were harvested and the data recorded. Observations of lethality, shoot production per explant, and overall vigor of explants were recorded. Results were analyzed based on average number of shoots per explant and overall vigor. Means were separated using LSD at $\alpha = 0.05$. From these data, the optimum treatment was determined.

The MS medium was not suitable for all treatments. Most explants either failed to produce shoots or died over the course of the experiment. Data from the leaf explant experiments were not presented as the treatments in this project produced neither shoots nor roots during the course of this experiment. All treatments of leaf explants with 35.5 μM BA reached 100% lethality by the date of harvest. No significant differences in performance or lethality were observed between the remaining leaf explant treatments.

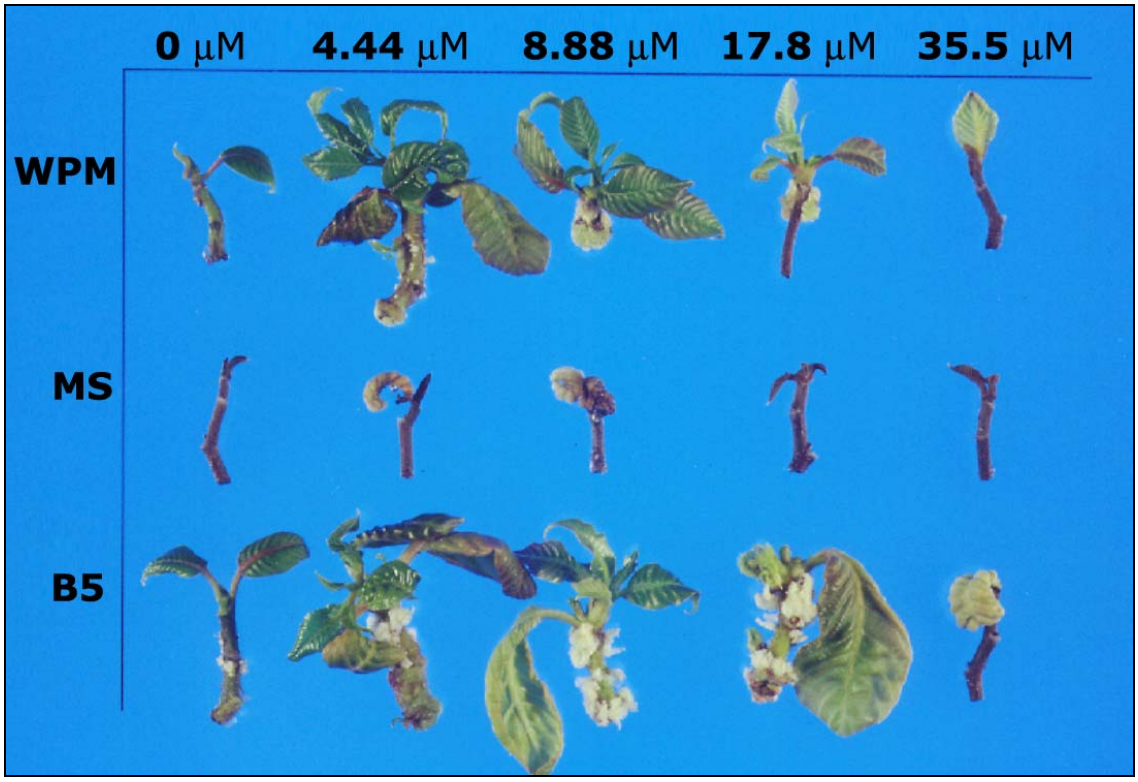

Fig. 5. Visual observation of representative shoot explants cultured under the tested factorial showed the gradation of effect the BA concentration (horizontal axis) had on the explants per each medium (vertical axis). Lower BA concentrations did not tend to produce callus or shoot multiplication while higher concentrations often resulted in necrosis and death of the explant. The MS medium treatments were not effective in multiplication of *Camptotheca acuminata*.

Shoot tip explants served as an efficient source of plant material for multiplication of *C. acuminata* (Fig. 5). Data collected from the two replicates in time were analyzed based on lethality, shoot production, and vigor (Table 3). The tested treatments significantly affected ($\alpha = 0.05$) shoot production by the shoot tip explants.

Table 3. Variable treatment effects on lethality, vigor, and shoot production in the micropropagation of *Camptotheca acuminata.*

Treatment	Lethality	Vigor Rank	Shoot Production[x]
B5-0 µM BA	0%[z]	7[y]	1.00 ± 0.00[w] bcd[v]
B5-4.44 µM BA	0%	4.5	2.20 ± 1.01 ab
B5-8.88 µM BA	0%	3.5	2.53 ± 0.91 a
B5-17.8 µM BA	0%	4	1.67 ± 0.72 bcd
B5-35.5 µM BA	26.7%	10	0.80 ± 0.56 bcd
MS-0 µM BA	66.7%	12.5	0.33 ± 0.49 d
MS-4.44 µM BA	21.4%	10	1.00 ± 0.68 bcd
MS-8.88 µM BA	66.7%	12.5	0.47 ± 0.74 cd
MS-17.8 µM BA	100%	14.5	0.00 ± 0.00 d
MS-35.5 µM BA	100%	14.5	0.00 ± 0.00 d
WPM-0 µM BA	0%	7	1.00 ± 0.00 bcd
WPM-4.44 µM BA	0%	1	2.73 ± 1.03 a
WPM-8.88 µM BA	6.67%	2	2.33 ± 1.29 ab
WPM-17.8 µM BA	6.67%	7	1.73 ± 0.96 abc
WPM-35.5 µM BA	33.3%	10	0.87 ± 0.74 bcd

[z] Means for n = 223, Explants not surviving at time of harvest
[y] Rank of treatments based on observed vigor; 1 being greatest observed vigor
[x] Treatment significant at $p \leq 0.0001$
[w] Means for n = 223, Shoots produced per explant.
[v] Mean separation within column at $\alpha = 0.05$ by LSD

Lethality

The average lethality per treatment was calculated as the percentage of explants not surviving at the time of harvest. The B5 and WPM media produced minimal lethality in the shoot tip explants even as the applied BA concentration rose. Conversely, the MS treatments produced severe lethality at almost all BA levels rising to 100% at both the 17.8 μM and 35.5 μM levels (Table 3).

Vigor

Vigor observations were also collected as a means of distinguishing between statistically and practically advantageous treatments (Table 3). Each treatment was visually compared to all others based on the following guidelines:

- Bright green color
- Absence of vitrification
- Actively growing and multiplying cultures
- Uniform growth with no excessive callus
- Absence of exudates into media

A sequential numeric rank was assigned to each treatment and averaged over the replicates. This vigor rank was used in the final recommendations of the optimal treatment combination. Ranks 1-5 were generally characterized by each of the positive points above. Ranks 6-10 indicated non-uniform growth and were often characterized by vitrification, pale green or yellow leaves, and some exudates. Ranks 11-15 were often necrotic or dead, had yellow or no leaves, and produced exudates in the media (Fig. 5).

Shoots per Explant

The treatment producing the highest number of multiple shoots was WPM with 4.44 μM BA, with 2.73 shoots per explant on average at harvest. Continued observations of these explants subcultured on the same medium showed that this medium was a successful means of multiplying *C. acuminata in vitro*, after this establishment study. Data for shoots per explant were not found to be normally distributed ($p \leq 0.0001$) using the Shapiro-Wilk and Kolmogorov-Smirnov tests.

The BA concentration in the treatment media significantly affected shoot production at $p \leq 0.0001$. Increasing concentrations of BA up to the 17.8 μM treatment increased the proliferation of lateral shoots (Fig. 6). This finding follows the known ability of cytokinins such as BA to break apical dominance in plants thus encouraging lateral and adventitious shoots (Coenen and Lomax, 1997; Jones, 1967). This result is vital in an *in vitro* plant multiplication scheme.

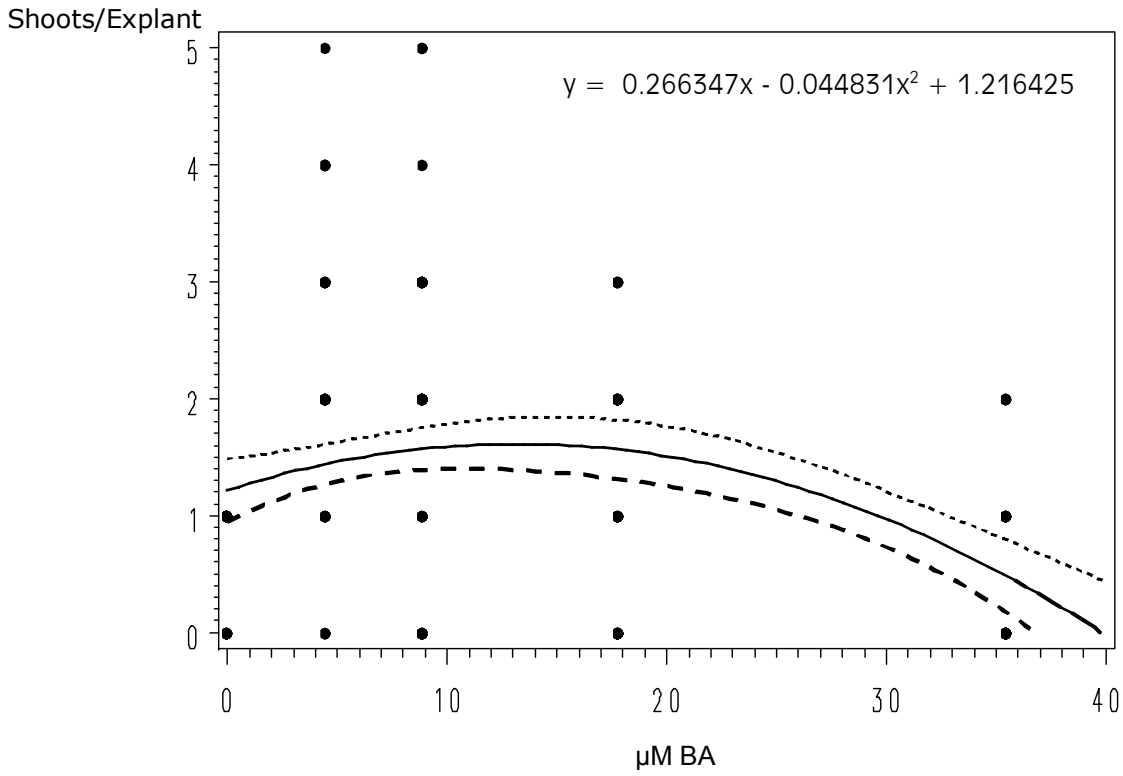

Fig. 6. Average number of shoots produced per explant of *Camptotheca acuminata* as affected by BA concentration in micropropagation medium. The regression curve and 95% confidence interval of shoot production per explant over increasing BA concentration indicates saturation between 8.88 µM and 17.8 µM.

Regression analysis of shoot production data was performed to determine the actual saturation point the increasing BA concentration had on the shoots per explant recorded during the experiment (Fig. 6).

The basal medium used in the treatments also significantly ($p \leq 0.0001$) affected the shoot production of the explants (Table 4). Both WPM and B5 media were adequate in the micropropagation of *C. acuminata*.

Plotting the effects of both medium and BA concentration graphically verify the visual observations (Fig. 5) indicating the best treatments occurred with WPM and B5

media and 4.44 μM and 8.88 μM BA (Fig. 7). Analysis of variance results confirm these observations indicating a significant interaction between the medium and BA concentration ($p \leq 0.0001$).

Table 4. Effect of basal medium on *in vitro* shoot production of *Camptotheca acuminata.*

Medium [z]	Shoot Production
WPM	1.73[y] ± 1.15[x]
B5	1.64 ± 0.98
MS	0.36 ± 0.61

[z] Variable significant at $p \leq 0.0001$
[y] Means for n = 208, Shoots produced per explant.
[x] Standard deviation

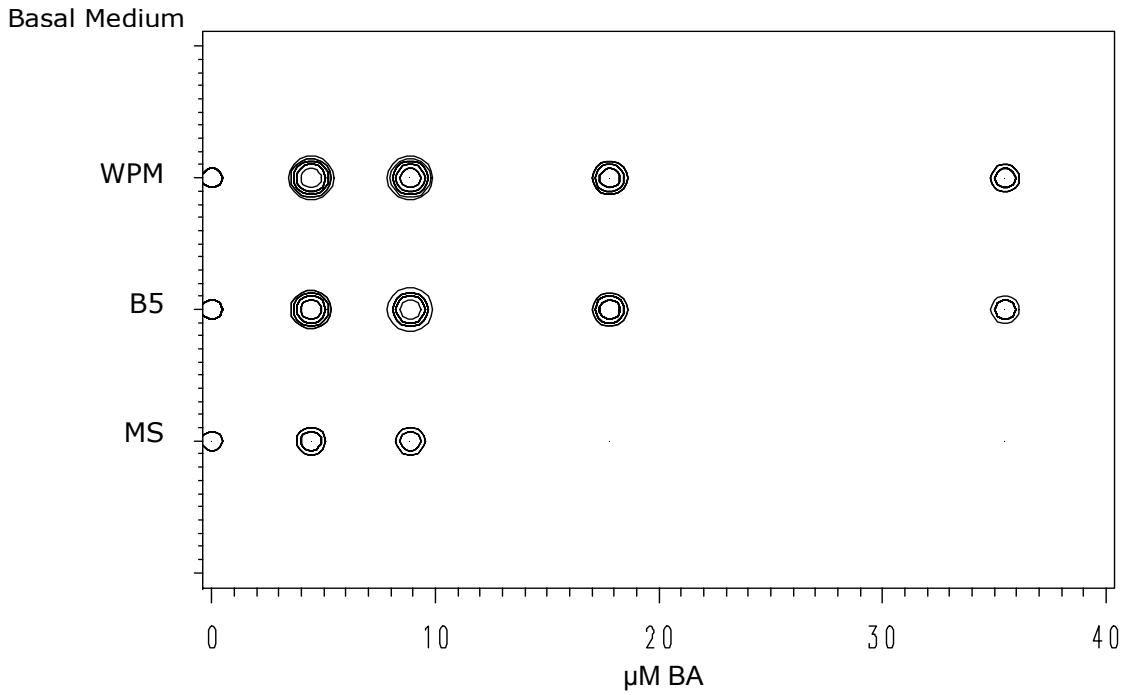

Fig. 7. Shoot production of *Camptotheca acuminata* as affected by basal medium and BA concentration. The bubble plot graphically indicates greatest shoot production per explant at the 4.44 µM and 8.88 µM BA concentrations with the WPM and B5 media.

Establishment

In vitro rooting trials resulted in highest rooting percentages and number of roots per microcutting at the 4.44 µM IBA treatment (Fig. 8). This treatment averaged 72% rooting and 1.96 roots per microcutting from the two replicates tested. Significant differences of roots per microcutting were found between all four treatment concentrations (LSD $\alpha = 0.05$). However, the 0.49 µM and 4.92 µM did not produce significantly different rooting percentages.

Visual observations noted good root formation at the 0.49 µM and 4.92 µM treatment levels and excess callusing at the 9.84 µM concentration. Roots formed at the 4.92 µM treatment typically arose from callus balls at the base of the microcutting while

this callus formation was usually absent in microcuttings treated with 0.49 µM IBA. Adequate survival (81%) was obtained through the acclimation process to greenhouse cultural conditions.

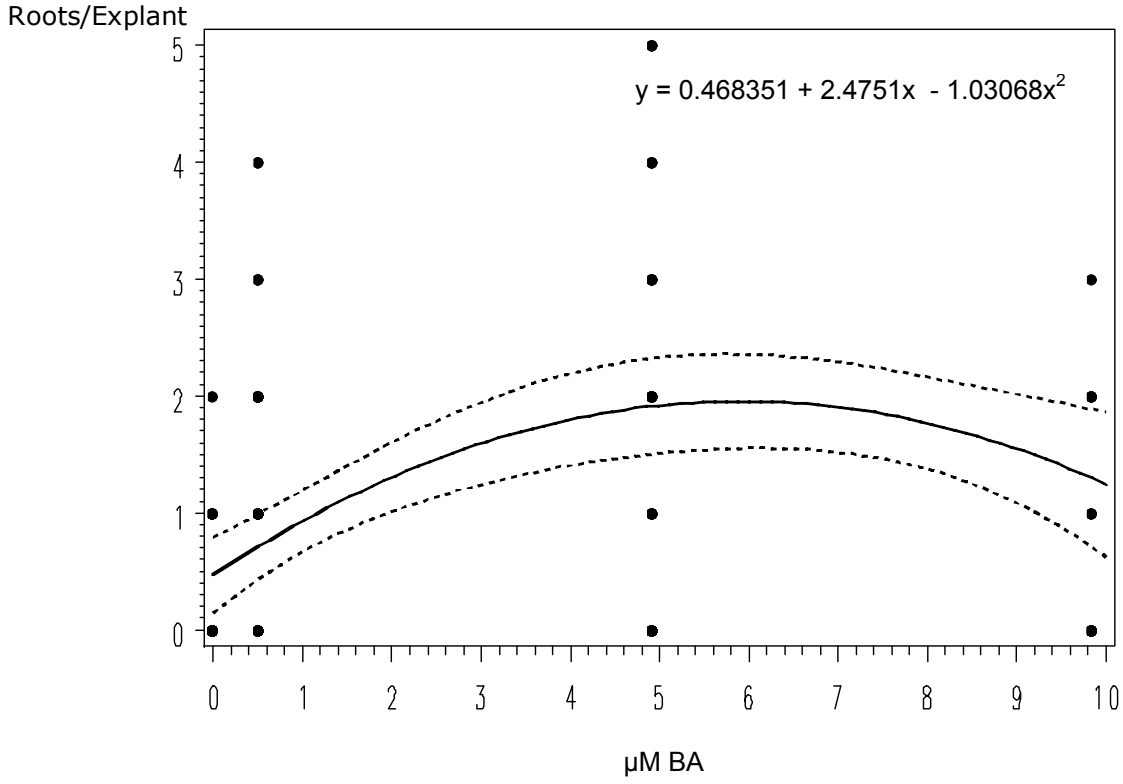

Fig. 8. Effect of IBA concentration on roots produced per microcutting rooted *in vitro*. Regression curve and 95% confidence interval of *in vitro* rooting trials indicates optimum IBA treatment near 4.92 µM.

Conclusion

The two highest producing treatments were WPM-4.44 µM BA and B5-8.88 µM BA. While the average number of shoots per shoot explant was the same, the recommendation is to use WPM with 4.44 µM BA. This conclusion is based on the

higher vigor observed with WPM-4.44 μM BA in both replicates, less variation seen with WPM-4.44 μM BA compared to B5-8.88 μM BA, and less BA plant growth regulator required in WPM-4.44 μM BA (decreasing cost in a commercial application).

The *in vitro* rooting and establishment trials indicated WPM with 4.92 μM IBA is the most effective means of rooting microcuttings of *C. acuminata* after clonal propagation; however, other rooting trials conducted *ex vitro* without application of growth regulators produced similar results (78% rooting success, unpublished). Thus, either system may prove useful in a commercial multiplication scheme, and further research is recommended to discern differences between the two methods.

Experiment 3 – Seed Storage of *Camptotheca acuminata*

To determine the effect of the storage treatments, germination percentages of each treatment were calculated and compared to each other as well as to the initial germination percentage of that seed crop. Vigor observations were also recorded to note any malformations or other detrimental effects of the storage treatments.

The results of this experiment showed the ability of cold storage to maintain and increase the germination percentage of *C. acuminata* seed. Initial germination percentages of harvested seed averaged 66% (non-transformed). Perdue and Smith reported similar percentages (59% and 78%) of new crop seed, varying by source (1970).

The unsealed control treatment showed no significant difference in germination from the sealed control; thus, the unsealed control has been removed from further

reporting. Insignificance of vessel sealing was also reported by Verapong-Suangtho (1992).

The two cold storage treatments were significantly distinguishable ($\alpha = 0.01$) from the control at 1.5 years of storage and from each other at 2 years (Table 5). Storage temperature significantly affected germination percentage in all trials. At extended storage over one year, this factor became increasingly significant.

Table 5. Average germination percentages of all harvest dates indicated that the cold storage treatments exhibit maintenance of germination percentage.

Treatment	0.5 Year	1.0 Year	1.5 Years	2.0 Years
-18/-20 °C	51.4 ± 5.82 [z] a[y]	46.9 ± 19.9 [z] a[y]	59.0 ± 3.84 [z] a[y]	68.9 ± 4.69 [z] a[y]
0/4 °C	46.1 ± 9.49 a	45.4 ± 23.6 a	73.9 ± 15.1 a	43.1 ± 7.29 b
Control	45.5 ± 5.93 a	29.2 ± 23.1 a	25.2 ± 4.16 b	0.00 ± 0.00 c
Significance [x]	**	**	***	****

[z] Percent germination (arc-sin transformed) means for all replicates; initial transformed germination average = 54.35%
[y] Mean separation within columns at $\alpha = 0.01$ by LSD
[x] **, ***, **** Significant at $p \leq 0.5$, $p \leq 0.001$, or $p \leq 0.0001$ respectively

Data over one year in storage is from a single harvest year, 1997, as the course of this research did not allow for long-term analysis of the other two harvest years. In each harvest year, the cold storage treatments produced greater germination percentages than the control (Table 5). Combined data of all harvest years illustrated a steady decline in percent germination in the control while both cold storage treatments maintained or

increased percent germination (Fig. 9). Percent germination data were found to follow a normal distribution using the Shapiro-Wilk and Kolmogorov-Smirnov tests.

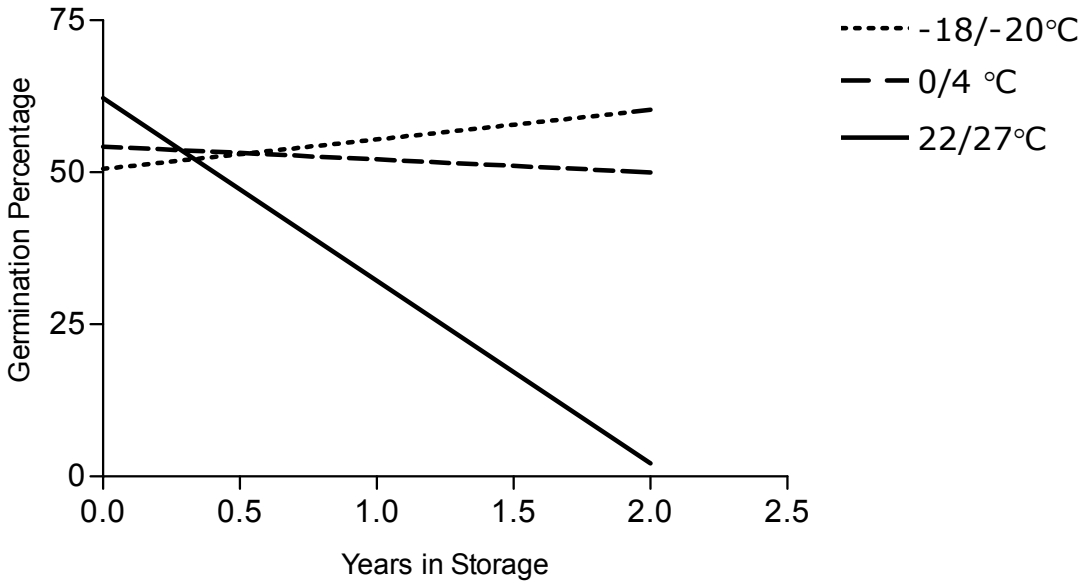

Fig. 9. A composite of regression lines showed the decrease in germination percentage (arc-sin transformed) with the control (room temperature) treatment and the maintenance or increase in germination percentage when seed were held in cold storage. Combined data of all harvest years was analyzed with linear regression.

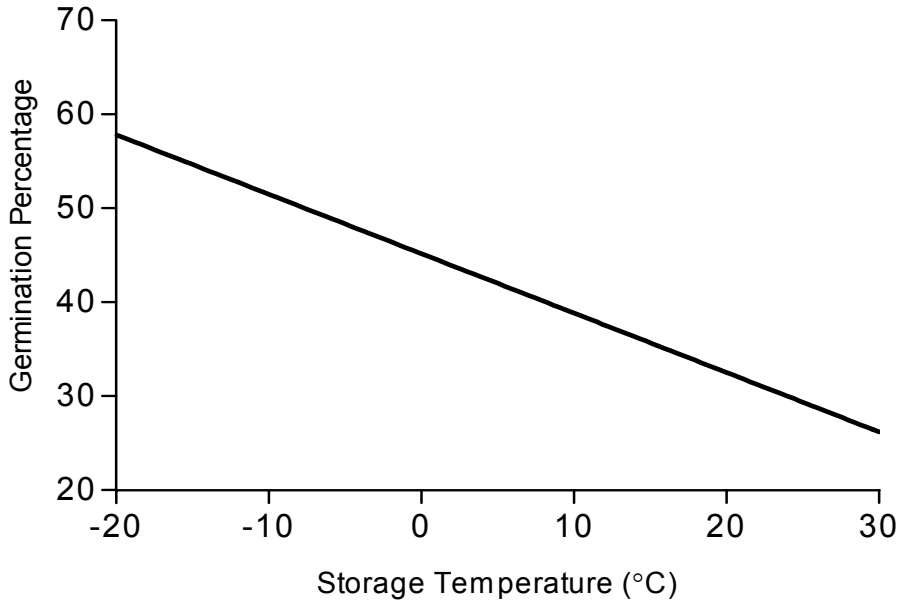

Fig. 10. Regression line of combined arc-sin transformed germination percentage data showed the effect of storage temperature treatment on germination of *Camptotheca acuminata* seed.

Regression analysis of combined germination percentages (arc-sin transformed) as an effect of storage temperature treatment exhibited decreasing germination percentage as the storage temperature increases (left to right). A maximum germination percentage was found with the cold storage treatments, and the colder treatment (left) resulted in greater numeric percentages (Fig. 10).

By separating the different harvest years to account for differences in time in storage, results showed an increase in germination percentages of cold stored seed on average over that of the control treatment. Regression analysis showed a maximum germination percentage higher than the initial transformed average of 54.4% when exposed to temperatures between 0 and –10 °C for 1.5 years. Also evident was the need

to decrease storage temperature from the 0/4 °C treatment to the –18/–20 °C treatment when storing longer than 1.5 years.

Fig. 11. Visual observations in this photo of a single replicate at 1.5 years in storage reinforced the finding that cold storage of seed can maintain germination percentage in *Camptotheca acuminata.*

Conclusion

From this experiment, it is concluded that cold storage of *C. acuminata* seed can maintain and possibly increase percent germination. Cold storage treatments of 0 to 4 °C and –18 to –20 °C produced significantly higher germination percentages than the room temperature control as well as the initial germination percentage calculated for that harvest year. Visual observations of seedlings indicated no significant detrimental effect

on seedling health of the cold storage treatments compared to new crop seed over the tested treatment levels and time in storage (Fig. 11).

These results show that cold storage of *C. acuminata* seed can be an effective means of prolonging germination percentage over time and indicate that germination percentages of this species may benefit from stratification. This finding agrees with findings by Perdue and Smith (1970) in trials of moist stratified cuttings stored at 4.4°C for 14 days.

SUMMARY

This research was designed to study and optimize the propagation of *C. acuminata*. This is a vital step in the ability to utilize this species in commercial production, as well as multiplying any mutant lines that arise from mutagenesis studies.

Experiment 1 tested the usefulness of a traditionally practiced K-IBA quick dip on the rooting of softwood cuttings. The application of a 5 second quick dip of 29.0 mM K-IBA produced 81.7% rooting success and averaged 3.87 roots/cutting. The results showed favorable response to this application and it is recommended as an efficient and quick way to multiply the species under field conditions where an acceptable parent source is available. The observed ability of this plant to flush new growth quickly is further evidence that apical cuttings as used in this trial are an efficient propagule.

Experiment 2 improved the practicality of previously published micropropagation schemes for this species. A Woody Plant Medium basal medium with the addition of 4.44 μM BA produced favorable results of the treatments tested in this experiment. This treatment exhibited no lethality and averaged 2.73 shoots/explant. Vigor observations as well as rooting success show that this technique is a viable means of multiplying the species *in vitro*.

Experiment 3 showed that cold storage of *C. acuminata* seed can maintain germination percentage over 2 years. Cold storage between 0°C and –20°C can significantly increase germination percentages over initial percentages at a minimum storage of 1 year, while maintaining germination percentage under this period. A

maximum germination percentage was observed at 73.9% when seed were stored 1.5 years at 0 to –4°C.

To conclude, this research has hopefully increased insight into the propagation of this subtropical species. Commercial production of this crop should not be limited by the ability to propagate it, given the reported findings in both field and aseptic conditions. Further research into the believed stratification requirement is prompted by this study. As well, larger field studies into the application of these propagation techniques to commercial production is warranted.

While *C. acuminata* remains a viable, if not the only, source for the medicinal compound camptothecin and this compound continues to show promise in combating some of mankind's most devastating diseases, research into it's action and production shall continue. The research set forth in this paper documents one facet of the consortium of teams investigating this species.

With relatively little known about the propagation of this tree, this research delved into traditional propagation by softwood cuttings, optimizing a micropropagation scheme for mass production, and seed storage treatments to enhance germination when sexual propagation is utilized. The results are a feasible means of propagating the species under field conditions as well as under aseptic conditions. As well, the data produced from seed storage trials support previous research that this species may benefit from seed stratification. Ultimately, it is believed that propagation issues should not hinder commercial production of this species.

The promise to contribute to our fight against some of our world's currently incurable diseases, if even in treatment alone, remains the driving force behind research into this species. Using what is learned from this research and the research teams around the world, continued efforts advance the effectiveness, production, and understanding of *Camptotheca acuminata*, the Tree of Joy.

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