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**Studies in tissue-cultured *Paulownia tomentosa* :: phenotypic stability, ploidy status, acclimatization, and in vitro cold storage /**

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STUDIES IN TISSUE-CULTURED PAULOWNIA TOMENTOSA:  
PHENOTYPIC STABILITY, PLOIDY STATUS, ACCLIMATIZATION,  
AND IN VITRO COLD STORAGE

A Thesis Presented

by

Lakshmi Jagannathan

Submitted to the Graduate School of the  
University of Massachusetts in partial fulfillment  
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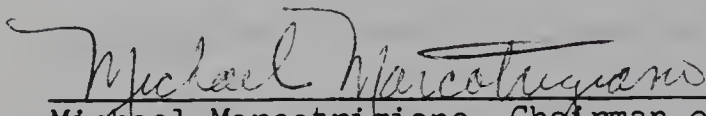
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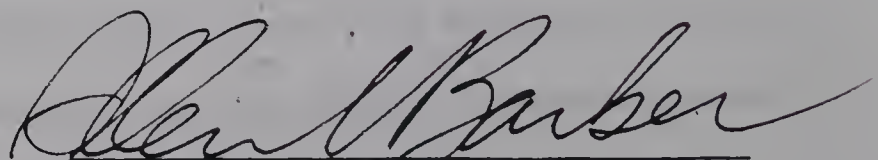
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# C H A P T E R I

## INTRODUCTION

Paulownia tomentosa Steud. (Empress tree) is a naturalized Asiatic tree which was first introduced into the United States in the nineteenth century (79). It is a fast growing deciduous tree in the Scrophulariaceae. Widely revered in Japan and utilized for its valuable wood, its commercial significance has been realized only recently in America (51, 79, 92). Besides its potential for export to Japan, it has been recognized for its suitability in strip mine reclamation (18, 101). This is due to its drought resistance and tolerance to high pH of the soil.

The tissue culture of woody plants has now become an extensive subject and has been widely reviewed (33, 121). The release from axillary bud dormancy is generally used to proliferate large numbers of shoots (19, 21, 42, 43). Recently, there have been reports of the production of adventitious shoots from other sources including callus (30, 45, 120).

Attempts at regenerating Paulownia plants via tissue culture, have been successful. Fan and Hu (34) regenerated shoots from nodal and internodal segments of P. taiwaniana, a closely related species. The organogenetic potential of hypocotyls was reported in P. tomentosa in 1983 (64). Rapid shoot multiplication from shoot tips was also obtained. In addition, the basal portions of cultured shoot tips gave rise to callus, which on occasion would give rise to shoots.

P. tomentosa is therefore a viable candidate for advanced in vitro manipulations.

Chromosomal changes and abnormalities contribute considerably to the phenotypic variability of tissue-culture regenerated plants. This instability is often a drawback, when propagating valuable clonal material. Yet, it can offer a high frequency and novel sources of variation, some of which may be favorable. This is particularly important in woody tree species where conventional breeding methods are slow. Since variability and stability of Paulownia tomentosa has not been previously investigated, this area has been considered suitable for study, especially with the characterization of any variants that might be obtained.

Successful multiplication does not ensure large numbers of surviving plants after transfer to ambient conditions. Studies aimed at maximizing the survival of regenerated plants are lacking in many important species, which can limit the advantages of micropropagation. Useful variants also need to be multiplied and successfully transferred to soil. Hence, devising techniques for maximizing survival rates is another objective of this research. To ensure similarity in the condition of plants being compared, roots from seedlings will be removed and the rootless shoots will be compared with rootless shoots grown in vitro. With a rootless control, the validity of the comparison would be increased over acclimatization experiments where well established, rooted seedlings are used as the control.

Storage of germplasm is an important consideration and tissue culture may offer a convenient alternative for this purpose. Long-term storage and maintenance of cultures in vitro is of immense practical value, and is therefore another aspect which deserves attention.

The ultimate goal of this research is to arrive at some conclusion about the practical application of tissue culture for the induction, isolation, and characterization of useful variants and for large scale propagation and storage of Paulownia tomentosa.

## C H A P T E R I I

### REVIEW OF LITERATURE

#### Phenotypic Stability and Ploidy Status of Tissue-Cultured Plants

Although mere ploidy status is not always a sufficient indicator of stability (85), changes in ploidy can have severe consequences on regenerated plants. The following review will be confined to the problem of changes in chromosome number. Stability in the chromosomal number of tissue-culture regenerated plants has been reported in several cases (7, 36, 119). In cultures of carrot (7) and celery (119), stability was attributed to the extent of tissue organization, since portions of the callus were maintained in a state of permanent differentiation.

The most common chromosomal changes are increases in chromosome number (2, 73, 108). Reduction in ploidy is less common. Reduction occurred in some regenerants from callus cultures of different species and varieties of scented geranium (90). Aneuploidy has been observed in 10-year-old rye-grass endosperm cultures (76) and in cell cultures of carrot (7), and oats (67).

Various factors can be responsible for variation in chromosome number. Major factors include the method of culture and the source of explant used. Regeneration from primary callus was shown to be conducive for producing wide variation in Durum wheat plants (9). The

widespread occurrence of polyploidy in plant tissue which was described by D'Amato (24) as "the typically chimerical nature of the plant body" can contribute to variation. As Reisch (80) expressed in his review of variability, if strict control of cell division and DNA replication, such as that normally occurring in cells of the shoot apex, could be attained, then stability would be ensured. Hasegawa (46) explained the stability of asparagus spears derived from shoot apex cultures to be due to the fact that they arose from axillary buds. Histological examination did not indicate the presence of any adventitious buds.

The culture medium can also have an influence on chromosomal stability particularly if media contains 2,4 - dichlorophenoxyacetic acid (2,4-D). Both 2,4-D and  $\alpha$ -naphthalene acetic acid (NAA) caused cytological disturbances in Vigna tissue cultures (52), with 2,4-D causing the highest ploidy and also the highest percentage of polyploid cells. Kinetin present in yeast extract affects ploidy level (106, 107). Torrey found that kinetin stimulated mitosis in endomitotic tetraploid cells which could have existed in vivo and it thus caused the selective production of tetraploid cells in pea root callus (107). The effects of para-fluorophenylalanine (PFP) on the ploidy status of regenerated plants has remained controversial. PFP is believed to favor the growth of haploid cells over diploid cells. Somatic reduction in chromosome number was induced by PFP in tetraploid grape (77) but no such effect was seen in cell suspension cultures of Datura innoxia (32). Griseofulvin (7-chloro-2',4,6-trimethoxy-6'-methyl-spiro (benzofuran-2(3H),1'-2 cyclohexene)-3,4'dione) is another chemical

reported to have the capacity to induce chromosomal instability. In plants of a petunia somatic hybrid regenerated from leaf sections, increases and decreases in ploidy as well as aneuploidy were seen (38).

Time in culture had an effect on the cytological composition in Daucus carota (7) cultures but not in Apium graveolens (119).

Selection pressure was considered to be the factor that resulted in the increase of diploid cells of Vicia hajastana cultures (86). Such pressure apparently counter balances disruptive processes which lead to the production of aneuploid cells. The selective advantage of diploid cells has been documented with Prunus amygdalus (68). Although variation in the number of chromosomes could be seen in cells of some of the calli, the organs isolated and plants regenerated were diploid. In contrast, tetraploid cells were selectively produced in pea root callus (107).

The cytological basis for variation in the number of chromosomes during culture has been studied. Bayliss (6) found factors such as partial spindle failure as evidenced by multipolar separations, lagging chromosomes and bridge formation by diconstrictional chromosomes to be responsible for variation in suspension cultures of Daucus carota.

Abnormalities in chromosomal number frequently have some deleterious effects. Callus tissues of pea completely lost their ability to initiate roots after years of subculture. This was concomitant with the occurrence of polyploidy and an increase in the frequency of aneuploidy (108). A problem associated with tetraploidy in tobacco plants regenerated from pith cells, was the inability to produce functional pollen (73).

Although chromosomal variability has its negative aspects, some of its consequences are useful. Larkins and Scrowcroft (59) have reviewed variability and stressed its potential for plant improvement in areas such as disease resistance, production of alkaloids, etc. They coined the term "somaclonal variation" to denote variation resulting from the process of in vitro culture, though this variation may not always be paralleled by chromosomal changes. Chromosomal instability can be used in breeding. McCoy (67) suggested that aneuploidy in oat could be useful in recovering recessive mutations. A good illustration of the benefits of tissue culture induced variation is the superior scented geranium cultivar 'Velvet Rose' obtained from callus cultures by Skirvin and Janick (89). They conducted extensive studies of somaclonal variation of several species and cultivars of Pelargonium (90). Aberrations occurred in plant and organ size, leaf and flower morphology, fasciation, pubescence, anthocyanin pigmentation, and essential oil constituents. Segregation of chimeral tissue, ploidy changes, and other chromosomal alterations were considered to be responsible. An interesting discovery was that the same variation occurred in the plantlets that were produced in the second cycle - from the tissue-cultured individuals derived from the original clones.

Phenotypic instability is often correlated with chromosome number changes. To cite an example, polyploid tobacco plants regenerated from pith cells had distinctive characteristics (73). They were rosetted, more pubescent, and had smaller, thicker leaves with prominent venation. Floral characteristics also differed from that of the

diploid plants. The appearance of sectorial chimeras for phenotypic characteristics has been attributed to cytochimerism (47, 66).

Chromosome number mosaicism was observed in shoot and root apices of in vitro regenerated Durum wheat plantlets (9). The authors suggested that the meristem originated from two or more initial cells. They conceded that the callus inducing medium may have caused chromosomal changes during mitosis in the process of in vitro culture. Yet, in contrast, even in the absence of changes in chromosome number, Gamborg et al (36) found changes in the growth habit and leaf morphology in plantlets regenerated from callus of mature embryos of sorghum. Some of the plants were also sterile.

Studies in tissue culture of plants are not always followed up by investigations of the phenotypic status of regenerated plants. However, there are some detailed reports comparing tissue-culture derived plants and those propagated by conventional asexual techniques. One point of interest has been the potential increase in yield. Swartz et al (100) found some increase in the vigor of the tissue-cultured plants of blackberry, as indicated by changes in leaf size, pattern, and increased yield. This increase was attributed to increased growth, since the number of fruits per unit length of cane did not increase significantly. In addition, only a single aberrant was observed. In strawberry, increased vigor and axillary bud activity increased yield (99). However, increase in flower density ultimately resulted in a reduction in fruit set and fruit weight because of competition, thereby reducing yield by weight per crown. In a Lilium hybrid maintained as a callus, a variegated variant was recovered.

Although its ornamental value was recognized, the plant was considered undesirable since it was low in vigor (94). Chrysanthemum plants regenerated from nine year old cultures exhibited variation in phyllotaxy, leaf, and floral morphology, and root growth (97). Lateral shoot growth was higher and numerous meristematic areas existed at the apex. Root growth and floral appearance was superior in those from short-term cultures. Thomas (102) observed a high degree of variation in growth habit, leaf morphology, and pubescence in a tetraploid cultivar of potato regenerated from shoot culture derived protoplasts. It was suggested that techniques for controlling variation be developed if variation was to be of agronomic use.

#### Transplantation Survival of Tissue-Cultured Plants

Unless adequate precautions are taken, survival after transplantation of micropropagated plants can be a problem as evidenced in rose (88), blackberry (16), and carnation (31). The vulnerability can be due to poor photosynthetic abilities (41), but more frequently is caused by the sudden decrease in ambient humidity after transplantation. Water stress has been seen to be one of the major drawbacks in the survival of in vitro cultured plantlets transferred from their high humidity environments (13, 88, 96).

In woody dicots, survival is frequently a problem, even after the adoption of post-transplantation measures (16, 61). This has prompted many researchers to investigate the factors causing death after transplantation. Suggestions have been made regarding the mechanisms

operating during the processes of acclimatization of tissue-cultured plants prior to transplantation.

Anatomical factors may be responsible for the susceptibility of in vitro grown plants to low humidity. Change in the development of epicuticular waxes is one such factor. Environmental factors have been known to affect wax development (1, 25). Baker (1) found that the effect was primarily on size, configuration, and distribution of the surface wax structures rather than on wax composition. Decrease in humidity or temperature and increase in radiant energy were all factors increasing wax development in brussels sprouts. Darnell and Ferree (25) also found that low soil water potential (water stress) increased development of leaf wax in field grown apple trees. However, decrease in temperature decreased wax quantity and light had no effect. Changes in wax composition were also observed. This difference in response of apple was attributed to variation between species.

Grout and Aston (40) attributed water loss through the cuticle to be the sole reason for poor survival of Brassica oleracea var. botrytis regenerated from meristem cultures. Contact angle measurements indicated a reduction in wax formation when leaves developed under high humidity. Scanning electron microscopy (SEM), contact angle measurements and quantitative measurements of wax indicated both quantitative and morphological changes in epicuticular wax on the foliage of plants of Brassica oleracea var. Currawong (39). Sutter and Langhans (96) suggested that changes in wax morphology occur in carnation plantlets regenerated from shoot tip culture. Their subsequent studies in cabbage (98) confirmed quantitative changes as

well. Transmission electron microscopy (TEM) indicated the lack of a well developed cuticle in sweetgum leaves from trees in culture (117). Further SEM investigations (118) aimed at determining if epicuticular wax deposits were responsible, showed that there was no variation in epicuticular wax morphology. However, these results did not preclude quantitative changes in wax.

Other anatomical characteristics may contribute to the water loss of aseptically cultured plants as observed in "Pixy" plum (13). The depth of the palisade mesophyll was significantly less in these plants when compared to the same plants after transfer to the greenhouse. The percentage of intercellular air space was also higher in the aseptically cultured plants. These two factors were considered to be responsible for the water loss. Leaves of cultured sweetgum (117) completely lacked palisade mesophyll although the field grown plants showed clear differentiation into spongy and palisade layers. TEM also showed large air spaces in the spongy tissue. Another feature accelerating water loss was protruding stomata. The almost complete absence of protective filiform hairs on the leaves of newly transferred plantlets was believed to have increased the likelihood of water stress in red raspberry (28).

Poor development of vascular tissues and roots may contribute to water loss of cultured plantlets. In sweetgum callus culture Birchem et al (10) reported formation of roots that lacked anatomical and morphological features of normal roots. The physiological function of these roots was therefore considered doubtful. Grout and Aston (41) reported restricted water movement due to the incomplete vascular

development between the roots and shoots of regenerated cauliflower plants. The successful adaptation of transferred plants involved the development of more substantial vascular connections.

Fuchigami et al (35) concluded that both lack of epicuticular waxes and stomatal response were causal factors in waterloss of aseptically cultured plum. Thus, physiological reasons can also contribute to water loss. Brainerd and Fuchigami (11) studied the process of acclimatization of aseptically cultured apple plants to low relative humidity to determine if stomatal functioning was involved. It was concluded that stomatal closure was slower in excised leaves of the tissue-cultured plants when compared to that of greenhouse grown plants, and therefore contributed to the greater rate of water loss. It was also noted that at corresponding percentages of stomatal closure, the rate of water loss was higher than that of greenhouse grown plants. It was suggested that this was due to higher cuticular water loss, higher stomatal frequency or larger stomatal apertures in the aseptically cultured plants. Subsequently, stomatal functioning in leaves of apple grown in vitro and in the greenhouse was studied in the presence of either darkness, mannitol, abscissic acid (ABA), or  $\text{CO}_2$  (12). Although the stomata closed in greenhouse plants in response to the addition of these factors, they generally remained open in the plants grown in vitro. Similar results were obtained for tissue-cultured cauliflower plants (114). It was observed that low K/Mg and K/Ca ratios and high levels of Na in the cauliflower guard cells interfered with movement of K into guard cells, thereby hindering stomatal closure.

A plausible approach to the problem of low survival rates, would be to adopt "hardening off" measures after transplantation. Although intermittent mist was effective in ensuring survival of micropropagated Dieffenbachia (78), it was not satisfactory for watermelon plants (4). Instead clear plastic cups were used to cover the plants. These cups were raised and then gradually removed for increasing lengths of time until the plants had adjusted to the environment. Such polyethylene covers and plastic frames were found to be effective in many cases (60, 83, 95). A polyethylene bag was also more suitable for Vanilla planifolia (58) since mist caused soft rot and death of all tissue-cultured shoots, including those rooted in vitro. Rootstocks of Prunus were successfully propagated by keeping them in humid propagating boxes in a shaded greenhouse after removal from the in vitro environment (53). Alternatively, McComb and Newton (65) used an anti-transpirant "Acropol" to increase survival of kangaroo paws (Anigozanthos sp.) rather than mere placement in a humid chamber.

The practice of rooting in vitro may increase future survival. Sometimes, as in Vanilla, it may be mandatory (58), while in other cases it may not be advisable. Hughes et al (49) found that Salpiglossis sinuata plantlets transferred to the soil failed to survive, because callus which existed between shoot and root served as an avenue for infection. It was necessary to use rootless shoots dipped in indole-3-butyric acid (IBA) and rooted in vermiculite. Davis et al (26) encountered the same problem in carnation and subsequently discontinued the process of rooting in vitro.

The other line of strategy to overcome problems in acclimatization is the adoption of a pre-transplanting regime. This would serve the purpose of hardening the plants and would obviate the need for a period of acclimatization after in vitro production. Wardle et al (114) described a method to increase surface wax on cauliflower leaves by decreasing humidity in vitro. Lanolin was spread on the agar medium and sterile packets of silica gel were suspended in the culture vessels. This combination caused severe desiccation. A lanolin layer alone was found to be sufficient to increase wax production and decrease the size of stomatal apertures. The only disadvantage was deleterious effects on growth, particularly that of the roots. Application of lanolin was therefore advised to be done after substantial growth had occurred. Another use of the lanolin layer was prevention of infection when lids were to be removed and plantlets were exposed to low humidity for hardening. Decreasing the ambient humidity by using  $\text{CaCl}_2$  helped to increase wax production in cabbage leaves (98). However, abnormal wax formations sometimes occurred. This was suggested to be due to poor mineral nutrition caused by dehydration of the agar. Brainerd and Fuchigami (11) discovered that satisfactory acclimatization could be accomplished by opening the vessels and exposing them to low humidity. Stomatal response was accelerated after a five day treatment of opening the vessels for periods of 0, 6, 12, or 24 hours with distilled water added to prevent the desiccation of the agar medium.

In some instances, both media and environmental alternations may be necessary. Hasegawa et al (46) reported the need for a specific

"pre-transplant culture" to avoid transplantation difficulties in asparagus. Removal of NAA from the culture medium was found to be essential to increase the vigor of roots and spears. It was also necessary to increase the light intensity to an optimum of 10,000 lux for ferning (differentiation of cladophylls) and also for increase in survival rate on transplantation. Higher photon flux densities were found to be helpful in acclimatization of rose (14). The effect of photoflux was considered to be independent of any temperature effect. In the propagation of head lettuce (57), it was found that transplantation shock could be alleviated by transferring the plantlets together with the intact agar medium. However, it was necessary to transfer them to sterilized vermiculite, since pathogenic infection becomes a problem.

There is an increasingly large number of species which are being propagated in vitro. For certain species, it may not be necessary to adopt the complex precautions suggested by some researchers. Simple post-transplanting care may be adequate. The need for protection would vary depending upon the susceptibility of different species of plants to dessication, their morphological status at the time of transplanting, and the rate of root and epicuticular wax development.

#### In Vitro Cold Storage

Germplasm storage in vitro has many advantages. The maintenance of disease-free and/or valuable germplasm are primary objectives (71). In

addition, frequent sub-culturing can be avoided (23) and space and labor requirements can be minimized (15, 71).

Cryopreservation is one method by which plant material can be stored indefinitely. For example, meristems isolated from shoot cultures of strawberry were precultured on medium supplemented with a cryoprotectant dimethylsulfoxide (DMSO), frozen at a cooling velocity of 0.84/min to a temperature of  $-40^{\circ}\text{C}$ , and stored in liquid nitrogen (54). This treatment resulted in 95% viability of the meristems after one week of storage. Even after eight weeks of storage, a considerable proportion of the meristems could be regenerated into plantlets.

Another technique is modification of the atmosphere. Low pressure and low oxygen (decreasing partial pressure of oxygen) storage of Nicotiana tabacum and Chrysanthemum x morifolium tissue cultures was attempted (15). Irrespective of the system used, growth decreased as the oxygen partial pressures decreased below 50mm Hg. This was found in both the differentiated shoot cultures of Chrysanthemum and the undifferentiated callus of tobacco. Plants regenerated from the stored cultures and subsequently grown, were normal and did not exhibit any phenotype differences.

The simplest and least expensive method of storage is slowing metabolism by cold storage at temperatures between  $1^{\circ}\text{C}$  and  $4^{\circ}\text{C}$ . Cultures can be conveniently stored in a refrigerator. Strawberry meristem plantlets could be thus stored for a period of six years (71). Plantlets periodically transferred to soil grew normally. Lolium multiflorum cultures could be stored at  $2-4^{\circ}\text{C}$  for 10-11 months

with 88% to 100% survival with losses due to contamination (23). A low light intensity was found to be necessary to maintain the green color of tissue cultures of Lotus corniculatus (105). Node cultures of lotus were incubated at reduced temperatures of 2-4°C for four weeks in culture media, with or without BA. There was 90% survival of plants from both media after transplantation. Proliferation rate was actually found to be higher for tissue-cultured apple shoots after a 12 month storage period (63). Shoots were stored at different temperatures: -17°C, 1°C, 4°C, and 26°C for periods of 3, 6, 9, and 12 months after which they were incubated at 26°C for one month for proliferation. Shoots stored at -17°C did not survive the thawing process and those maintained at 26°C had very poor survival rates because of contamination, dessication, and nutrient depletion. Storage temperatures of 1°C and 4°C were suitable for storage without loss of growth potential.

It can therefore be concluded that it is worthwhile to attempt cold storage of cultures following in vitro studies because of the simplicity of the technique and its practical value.

## C H A P T E R I I I

### MATERIALS AND METHODS

#### In Vitro Culture

Previously developed in vitro techniques for plant regeneration and shoot proliferation of Paulownia tomentosa were used with some modifications (64). The basal medium for all experiments consisted of Murashige and Skoog (MS) medium (Appendix A) (72), 0.8% Difco Bactoagar, and 30 g/l of sucrose, unless stated otherwise. The media was adjusted to pH 5.7 and autoclaved at 121°C for 15 minutes at 1.1 kg/cm<sup>2</sup> after the addition of all components.

Explants were cultured either in (a) 120 ml (5 cm diameter) wide mouth glass jars filled with 30 ml of medium and covered with plastic lids; (b) sterile disposable plastic petri plates (60 x 150 mm) filled with 10-12 ml of medium and sealed with parafilm; or (c) glass shell vials (95 x 25 mm) containing 10 ml of medium solidified at a slant. The cultures were grown at ambient room temperature of  $27 \pm 3^{\circ}\text{C}$  under cool white fluorescent light ( $.98 \pm .18 \times 10^4 \text{ mW/m}^2$ ) for a 16 hour light/8 hour dark diurnal cycle (= standard environment).

#### Shoot Proliferation

Seeds from a single Paulownia tomentosa tree growing in College Park, Maryland, were collected from dehiscing seed capsules and were manually dewinged to facilitate sterilization. Seeds were surface sterilized by swirling in a 5% AgNO<sub>3</sub> solution for 30 seconds, washed

in sterile water, and dried on sterile filter paper under a laminar flow hood. Approximately 100 seeds were scattered into vials containing half strength MS medium and germinated in the lighted environment described above. About 20 days after germination, seedlings were separated out and transferred, one per jar containing 30 ml of basal medium supplemented with 1.0 mg/l 6-benzylaminopurine (BA) and 0.1 mg/l indole-3-butyric acid (IBA). After 21 days of culture, shoot tips were dissected apart by stripping off all but the two uppermost leaves and placed vertically in jars containing basal medium without IBA, but with 3 mg/l BA to increase shoot proliferation. Shoot cultures were maintained by transferring 10-15 shoot tips to jars containing fresh media at 21 day intervals.

#### Organogenesis of Hypocotyls

Seeds of P. tomentosa were germinated as before under sterile conditions. Eighteen days after sowing, seedlings were transferred to the dark for three days to elongate hypocotyls. Excised hypocotyls were transferred to petri plates containing 10-12 ml of MS medium plus indole-3-acetic acid (IAA) and 6-furfurylaminopurine (kinetin) each at 3 mg/l. Eight hypocotyls were placed in each plate. After about 30 days, individual shoots regenerated from the hypocotyls (Figure 1) were removed as and when they were produced, and were placed individually in vials containing basal medium with IBA at 1 mg/l to induce rooting.



Figure 1. Shoot production from hypocotyl segments of Paulownia tomentosa.

## Phenotypic Stability and Ploidy Status

Shoots regenerated from hypocotyls were coded to trace their origin to a particular hypocotyl explant. When the rooted shoots were at least 2 cms long, shoot tips were removed and rooted in distilled water in 15 x 45 mm micro-sample vials. After roots were removed for fixation for cytological analysis, shoots were rerooted and transferred to the greenhouse. A seedling population for comparative study was also transferred to the greenhouse. All the plants were grown under natural daylength in a polyethylene greenhouse ( $42^{\circ} 22.6'N$   $72^{\circ} 31.31'W$ ) vented at  $25^{\circ}C$  with a minimum night temperature of  $18^{\circ}C$ .

### 1. Phenotypic Analysis

The following observations were recorded for 75 randomly selected hypocotyl-derived plants and a corresponding number of seedlings. Leaves chosen for measurement were the first set of fully expanded leaves below the terminal shoot apex.

1. Length of guard cells measured with a light microscope and a micrometer at a magnification of 120 X. Ten observations were made for each plant and the mean calculated.
2. Stomatal frequency/unit area. The number of stomates in a given area ( $0.0132 \mu m^2$ ) measured by the micrometer was determined. The mean of five observations made for each plant was used.
3. Number of serrations/leaf.
4. Number of major lateral veins/leaf.

5. Length/width ratio of the leaf blade.

6. Petiole length.

In addition, obvious morphological deviations were recorded qualitatively.

## 2. Cytological Analysis

A suitable technique was selected for metaphase analysis of root tip cells. The chromosome number of  $2n = 40$  previously determined using pollen mother cells (115) was confirmed using seedling root tips. Roots were treated for one hour in 0.05% colchicine, placed in distilled water for two hours, transferred to a fixative of 3:1 absolute alcohol-glacial acetic acid and stored at  $4^{\circ}\text{C}$ . After 48 hours in the fixative, roots were transferred to micro-sample vials and stored in 70% ethyl alcohol at  $0^{\circ}\text{C}$  for later use.

Root tips were excised from the fixed roots, placed in a watchglass containing a 9:1 volume mixture of 1% aceto-orcein and 10% HCl and heated for a few seconds over an alcohol lamp. Root tips were then squashed in 1% aceto-orcein on a slide by applying firm pressure to spread the cells. Edges of the coverslip were sealed with melted paraffin. Chromosome counts were made from these temporary slides using an oil immersion objective at a magnification of 2800 X.

## 3. Electron Microscopy

Leaf samples from a normal green tissue-cultured clone of Paulownia and from variegated and albino regions of a variegated tissue-cultured clone were prepared for transmission electron microscopy using

previously devised techniques (64). Stained sections were examined immediately with a Zeiss EM 9S-2 transmission electron microscope (Thornwood, New York) at an accelerating voltage of 60kV. Standard photographic methods were employed.

### Transplantation Survival

Two sets of experiments were conducted to develop transplanting techniques for micropropagated shoots, when a sufficient number of shoots were proliferated in vitro,

#### Experiment 1

Seeds were germinated in the greenhouse to obtain seedlings that could be compared with the in vitro grown shoots. About 21 days after germination, when they were approximately the same size as the tissue cultured shoots, 300 seedlings were randomly selected and their shoot tips were removed to make them similar to the tissue-cultured shoots. Seedlings were transplanted into 19 x 10.5 cms clear plastic boxes containing commercial starting media. Three replicates (boxes) each consisting of 50 seedling shoots were placed uncovered under intermittent mist in the greenhouse at a temperature of  $28^{\circ}\text{C} \pm 3^{\circ}\text{C}$ . Three replicates were covered with clear plastic lids and placed under cool white fluorescent lights with the temperature of media in the boxes being  $25^{\circ}\text{C}$ . Similarly, in vitro grown shoots were separated out and placed under the same conditions in a mist house or under lights. The experiment was replicated two times, four weeks

apart. After a period of 17 days, the percentage of survival and total dry root weight was recorded for each replicate (box). The number of primary roots on each shoot was also counted. Means were calculated for the two replications.

### Experiment 2

Some of the in vitro grown shoots were rooted in vitro in jars containing MS media with 0.1 mg/l IBA. Pre-rooted shoots were transplanted into plastic boxes with the same soilless media and kept under mist or placed covered under lights. Rootless tissue-cultured shoots were transplanted in the same way for comparison. After 17 days, the percentage of survival for each replicate was recorded and the mean for two replications calculated.

The results of both these experiments were statistically analyzed by analysis of variance to determine the effects of in vitro culture, environment, and rooting in vitro on the survival of the transplanted shoots.

### Cold Storage

To obtain a standard rate of shoot proliferation in vitro (number of shoots produced/shoot tip), several jars containing 15 shoot tips each were grown for 21 days. Subsequently, the shoots were dissected apart and the mean number of shoots was determined.

### Experiment 1

Jars, each containing 15 shoot tips, were maintained under lights in the standard environment for 21 days. After proliferation they were stored in the dark in (a) a refrigerator at a temperature of 4°C, or (b) a cold storage room with the temperature at 7°C  $\pm$  2°C. After 21 days of storage, one jar was removed from each cold storage environment. Shoot tips were excised and placed at the rate of 15/jar in other jars containing shoot proliferation media. These were kept in the lighted environment at room temperature. The rate of shoot proliferation was determined after 21 days. This procedure was repeated on cultures which were stored for 42 and 63 days.

### Experiment 2

Jars containing 15 freshly dissected shoot tips were immediately placed in storage at 4°C or 7°C. After 21, 42, 63, and 84 days of storage, four jars were removed subsequently from each storage environment and kept in the standard environment to observe shoot proliferation.

C H A P T E R I V  
RESULTS AND DISCUSSION

Phenotypic Stability and Ploidy Status  
of Tissue-Cultured Plants

Phenotypic Stability

The mean length of guard cells was 0.24  $\mu\text{m}$  for both the tissue-cultured (TC) population and the seedling population (SG) (Table 1). Stomate frequency was 5.05 and 4.79 in a leaf area of 0.0132  $\mu\text{m}^2$  for the TC and SG shoots respectively. These values did not differ significantly between the populations. The variance within each population was not significantly different for either of the two measurements. With respect to the other leaf measurements, the means for the number of serrations/leaf and the number of major lateral veins in each leaf did not differ significantly. The ratio of leaf length/width also did not vary significantly, and homogeneity of variance was established for the two populations. The TC shoots showed a highly significant increase in the ratio of leaf length to petiole length, a function of decreased petiole length (Table 1).

Measurement of guard cell size was used for the detection of gross changes in ploidy. This technique has been utilized as an indicator of such changes (24, 48). In kale, stomata were not only enlarged in the polyploids but atypical triangular stomata were sometimes present (48). In cytochimeras of tomato (91), however, guard cell size indicated the ploidy level of only the L1 histogenic layer (i.e., the

TABLE 1

LEAF MEASUREMENTS OF HYPOCOTYL-DERIVED SHOOTS  
AND SEED PROPAGATED SHOOTS OF PAULOWNIA TOMENTOSA

Measurement <sup>y</sup>	Population		
	Hypocotyl- derived	Seedling	P
Guard Cell Size ( $\mu\text{m}$ )	0.2	0.2	NS <sup>z</sup>
Stomate Frequency (number/ $0.0132 \mu\text{m}^2$ )	5.0	4.7	NS
Number of serrations/ leaf	68.4	63.9	NS
Number of major lateral veins/leaf	13.3	13.7	NS
Leaf length/width ratio	0.9	0.9	NS
Leaf length/petiole length ratio	1.6	1.1	***

<sup>z</sup>Nonsignificant (NS), or significant at 5% (\*), 1% (\*\*),  
0.1% (\*\*\*) level.

<sup>y</sup>Mean of 75 shoots.

epidermis). Although mean guard cell size of the TC population was not significantly different from the SG population, this does not preclude the possibility of the existence of polyploids. The uniformity between TC and SG plants as indicated by the Paulownia leaf length/width ratio was similar to the situation in tissue-cultured strawberry plants (99). The leaf length/petiole length ratio was significantly higher in the TC plants of Paulownia. The reverse, a decrease in this ratio occurred in tissue-cultured strawberry plants, where the increased petiole length was considered to be a reflection of increased vigor. In Paulownia the difference seen is probably a consequence of initial physiological stress rather than any permanent heritable change in morphology. On the whole, the population could be assessed to lack the variability in leaf characteristics sometimes reported in tissue-cultured plants (99, 100). This uniformity is in contrast with the extensive variation in leaf morphology reported for scented geranium (89) and that of Chrysanthemum regenerated from long-term cultures (97).

It is to be emphasized that the TC shoots in the Paulownia experiment here are of adventitious origin and distinct from those proliferated from the excised shoot tips. Polysomatism occurs in differentiated plant cells and they can therefore exhibit endopolyploidy. This can result in chromosome number mosaicism of the original explants. Consequently, in the absence of a selection pressure against non-diploid cells, the adventitious shoots could exhibit chromosomal variability. This factor was responsible for instability of kale (Brassica oleracea L.) plants regenerated from

stem pith explants (48). In Paulownia, however, the hypocotyl-derived plants generally appeared uniform. Further long-term studies under field conditions would be useful, as they would allow the general morphology, growth rates, flowering, and fertility to be evaluated.

### Ploidy Status

Since the shoots regenerated from hypocotyls are adventitious, an assay of the genetic stability of the clones is essential. Ideally, karyotype analysis is desirable. However, as Renfroe et al stated (81), accurate karyotypes are difficult to obtain in tree species with high numbers of small chromosomes. In fact, the problems encountered in obtaining sufficient numbers of suitable mitotic figures, led Renfroe et al to utilize cytophotometry for measuring nuclear DNA content of in vitro regenerated adventitious shoots of Pinus taeda (loblolly pine). The difficulty in obtaining cytological data in Paulownia also indicates that such a line of investigation is probably warranted.

Using pollen mother cells, the chromosome number of Paulownia tomentosa has been established as  $n=20$  (115). The attempt to establish this chromosome number in root tips of the standard seedling by using a staining and squashing technique was successful (Figure 2). However, difficulty was encountered in obtaining clean roots in suitable mitotic stages from tissue-cultured material. The large number and the minute size of the chromosomes were also responsible for precluding a detailed record of the chromosome numbers of all 75 clones of hypocotyl-derived shoots.

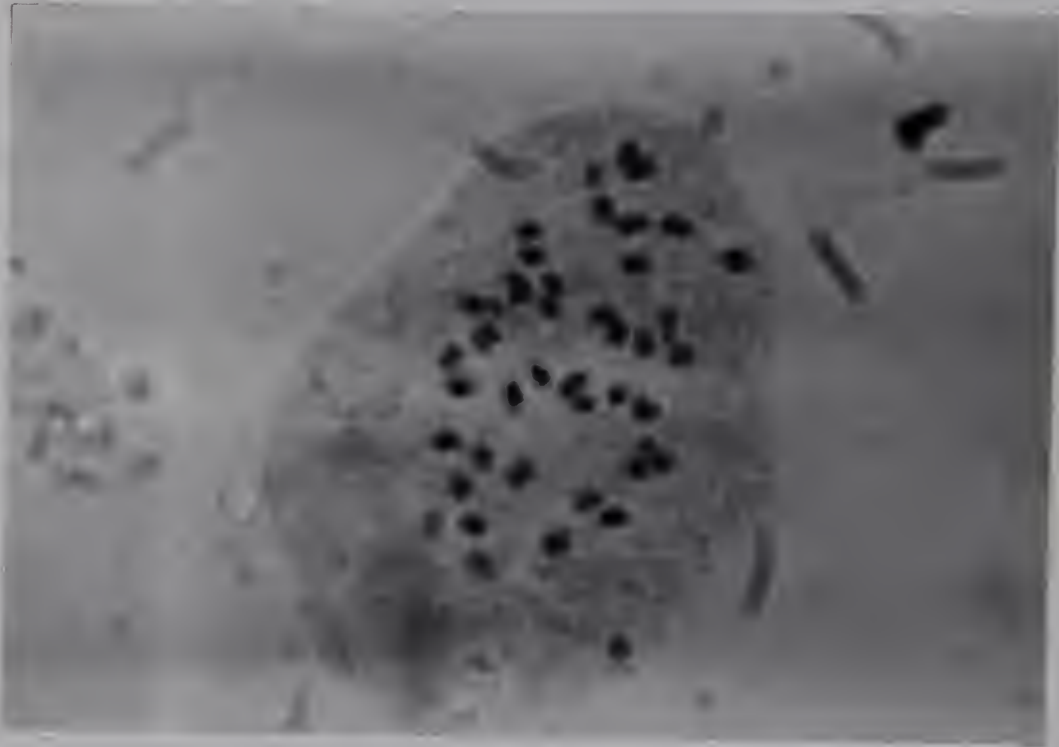


Figure 2. Mitotic root tip cell of a seedling of Paulownia tomentosa showing chromosome number of  $2n=40$ .

Metaphase counts made on some clones indicated no change in ploidy level (Table 2). In one clone (75d), some of the cells appeared to have reduced chromosome number while some had the normal diploid number. Thus, the possibility of cytochimerism exists. Mericlinal and periclinal cytochimeras were present among plants regenerated from anther and stem internode cultures of Lycopersicum peruvianum (91). The ploidy of the histogenic layers varied. Since adventitious roots normally arise from the "corpus" or L3, estimation of chromosome number from root meristems in a  $4n-2n-2n$  periclinal chimera would indicate diploidy even though the L1 was tetraploid. In the mericlinal tomato chimeras, variation was found between different shoots of the same plant.

Mixoploids were seen in barley plants regenerated from microspore callus (70). A preponderance of diploid cells in the root tips resulted in normal fully fertile plants whereas in individuals with a greater number of tetraploid and aneuploid cells sterility was high. A similar condition could exist in some of the clones of Paulownia. Due to the possibility of cytochimerism, a conclusive statement regarding the chromosomal number stability of all the TC plants cannot be made.

### Instability

Even though overall stability prevailed in the population of TC plants, a few phenotypic variants occurred.

1. Serration clone 63. Leaves have distinctly sharp serrations with each leaf having a pentagonal shape (Figure 3). Other characteristics appear normal. Chromosome number is  $2n=40$ . Changes in

TABLE 2

CHROMOSOME NUMBERS OF SOME HYPOCOTYL-DERIVED CLONES  
OF PAULOWNIA TOMENTOSA

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Clone Number	Chromosome Number (2n)
8d	40
10a	40
11d	40
13b	40
19c	40
23b	40
31a	40
34	40
39b	40
46c	40
58a	40
63	40
66c	40
77	40
81b	40
87b	40
108	40
120c	40
75d	20,40
24 (Variegated)	40

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## Figure 3

Variants from hypocotyl derived shoots of Paulownia tomentosa:  
(A) left to right, leaf from normal plant, leaf from serrated  
clone 63, leaf from dwarf clone 56b. (B) Flecked clone 59A.  
(C) Dwarf clone 56b.

A



leaf morphology are not uncommon in tissue-culture regenerants. Plants with lobed leaves and some with dentated ones were seen among regenerants from callus of Pelargonium sp. (90). These were associated with polyploidy and sterility.

2. Dwarf clone 56b. Leaves are thick and curled (Figure 3) and the plant is stunted and compact. Upon transfer to the greenhouse, an axillary shoot that was produced from it in vitro also has the same characteristics, confirming it to be a discrete variant and not the result of cultural and environmental factors. Dwarf plants which had lower yields were found in tissue-cultured strawberry (99). Tetraploid dwarf plants occurred in Pelargonium (90). Due to the difficulty in rooting clone 56b, ploidy level could not be determined in this variant. Guard cell size of  $0.026 \mu\text{m}$  was higher than the mean guard cell size ( $0.024 \pm .0001 \mu\text{m}$ ) of the population. This might be an indication of increase in ploidy as was the case in Pelargonium (90) in which ploidy level was correlated to stomate size. Further evidence is needed before drawing definitive conclusions.

3. Flecked clone 59a. This plant exhibits white and light green flecks on the leaves (Figure 3). Such conditions have been attributed to the presence of a white L1 histogenic layer (55). Hence this could be a mosaic or a periclinal chimera containing albino epidermal cells (derived from the L1 layer). Such cells can be infrequently displaced into the mesophyll of the lamina (93), resulting in light regions. Random flecking can also be caused by mutable nuclear genes (22).

4. Premature flowering clones. Five of the plants in the population, 25, 64a, 73a, 106, and 120e, produced flower buds which



Figure 4. A hypocotyl-derived early flowering clone.

developed normally and opened (Figure 4). Floral morphology and color were normal. No flowering occurred in the seedling population. This premature flowering is unusual considering the juvenility of the plant material and the time of bud opening. Paulownia derived from seed generally begin to flower in the third growing season. Flowering occurs in April after the dormant period (101), and not before going into dormancy as it did with the TC clones. Conditions maximizing growth such as that obtained by continuous placement in a greenhouse, can shorten the juvenile phase and result in early flowering as was seen in birch (62) and tea crabapple (123). However, the SG plants growing under identical conditions did not flower. Suggestions have been made regarding the physiological basis of phase change from juvenility to maturity and flower induction (44). Abscissic acid (ABA) has been implicated in the stabilizing of the mature form of Hedera helix by prevention of gibberellin (GA) induced reversion to the juvenile phase (82). At this point, only conjecture is possible regarding the explanation of this phenomenon in Paulownia. However, causes are most likely physiological rather than heritable. It is unlikely that five unrelated clones would display the same spontaneous mutation resulting in early flowering. The import of this early flowering is considerable because of the ease with which inheritance studies can be carried out and the implications concerning accelerated breeding programs.

5. Variegated clone 24. Prior to random selection of 75 hypocotyl-derived shoots for the phenotypic study, an important variant clone was observed in vitro. This appeared to be partially

variegated. It was separated out and proliferated to obtain additional shoots for observation. Axillary shoots that are irregularly variegated have been obtained as well as green shoots and shoots that are almost completely albino. The plant is easily rooted and can be grown in soil (Figure 5).

Cell lineage variegation occurs when variant areas can be traced to a common cell origin. They can be caused by plastome or nuclear (genome) mutation (55). Conclusive proof of plastome mutation has been obtained in several species (110, 111). Mutant chloroplasts in a variegated mutant of Nicotiana tabacum contained DNA that was different in composition from DNA in normal chloroplasts (122). Presence of "mixed cells" containing normal and abnormal plastids is morphological evidence for plastid mutation. Other indications are the occurrence of non-Mendelian ratios in the progeny of self-fertilized plants and maternal inheritance linked to the exclusion of plastid DNA from the pollen parent, as observed in many species (55). Maternal inheritance was evident in Hosta when reciprocal crosses were made between wild type green and variegated clones (110). Persistence of "mixed cells" was further proof of plastome mutation.

In the event that spontaneous or induced mutations occur in the proplastids of the shoot apical meristem, sorting out of normal and mutant plastids by successive cell divisions normally occurs (55). Stable periclinal chimeras can then be formed. Self fertilization, forcing out adventitious shoots from specific histogens and the examination of epidermal guard cells would be necessary to establish the nature of the different histogens of the periclinal chimera (104).



Figure 5. Hypocotyl-derived variegated clone 24.

Such a study of a plastid mutant is the work done by Miller et al with Daucus carota "Danvers" (69). A chlorophyll mutant induced by ethyl methanesulfonate (EMS) was histogenically analyzed and found to be a GGW (green L1, green L2, white L3) periclinal chimera with phenotypically white sectors of GWW. Prior to sorting out, "mixed cells" were found. Green and white plants were regenerated from callus cultures obtained from leaf and petiole sections.

Nuclear factors influencing chloroplast structure and function have been recognized (104, 116). Gene mutants were in fact found to be useful for learning about formation and functioning of chloroplasts and biochemical factors influencing the process (116). Nuclear mutation would be characterized by Mendelian inheritance unless there is abnormal chromosomal behavior (55). Breeding experiments showed segregation in ratios of 3:1 after selfing selected Pelargonium zonale chimeras (103).

The variegated clone 24 appears to be undergoing the process of "sorting out" and superficially resembles a typical plastid mutant. Examination of root tip cells showed diploidy with chromosome number equal to 40, indicating that ploidy changes were not responsible. The presence of white leaves and green leaves on the variegated plants is inconsistent with most descriptions of chlorophyll deficiencies controlled by the nucleus. Preliminary electron micrographs (Appendix B) seem to indicate destruction of chloroplast structure in leaf sections from albino shoots of the variegated clone. This appears to be similar to the condition observed in a plastid mutant of Hosta (110) where the high degree of vesiculation observed in the leaf cells was

attributed to chloroplast breakdown. In comparison, the normal leaf of Paulownia appears to have the greatest number of normal chloroplasts lining the cell wall, with the mosaically variegated leaf area having an intermediate condition (Appendix B). Further detailed study of the ontogeny of the chloroplasts is necessary. At present it is premature to draw any definitive conclusions about the cause of this variegation. Stabilization into a periclinal chimera would facilitate further investigations. Until plants begin to flower and inheritance studies can commence, the cause of this variegation remains speculative.

#### Transplantation Survival

The percentage of survival was maximum (100%) for the seedlings (standard method of propagation - ST) in both the clear plastic box and under intermittent mist (Table 3). This was significantly higher than the percentage of survival of the tissue-cultured (TC) shoots which was 89.3% for those grown in the plastic box and 73.3% for those under mist (Table 3). The analysis of variance showed that the environment did not have a significant effect on the percentage of survival.

The mean root weight per shoot was the highest for ST shoots in mist (1.9 mg) and the lowest for the TC shoots in the plastic box (0.4 mg) (Table 4). Both the method of culture and the environment had a highly significant effect on root weight (.0001 level of significance). The root weight was higher under mist. The interaction of these two factors was also highly significant, which means the

TABLE 3

EFFECT OF METHOD OF CULTURE AND ENVIRONMENT  
ON PERCENTAGE OF SURVIVAL OF TRANSPLANTED SHOOTS  
OF PAULOWNIA TOMENTOSA

Culture	Percentage of Survival <sup>y</sup> Environment	
	Plastic Box	Mist
Seedling	100.0	100.0
Tissue culture	89.3	73.3
Plastic Box vs. Mist	NS <sup>z</sup>	
Seedling vs. Tissue Culture	***	
Culture x Environment	NS	

TABLE 4

EFFECT OF METHOD OF CULTURE AND ENVIRONMENT  
ON ROOT DRY WEIGHT OF TRANSPLANTED SHOOTS  
OF PAULOWNIA TOMENTOSA

Culture	Mean Root Dry Weight (mg) <sup>y</sup> Environment	
	Plastic Box	Mist
Seedling	0.4	1.9
Tissue Culture	0.4	0.7
Plastic Box vs. Mist	*** <sup>z</sup>	
Seedling vs. Tissue Culture	***	
Culture x Environment	***	

<sup>z</sup>Nonsignificant (NS), or significant at 5% (\*), 1% (\*\*),  
0.1% (\*\*\*) level.

<sup>y</sup>Mean of 6 replications, with 50 shoots/replication.

effect of tissue culture on the root weight differed significantly under different environments. There were no significant differences in the mean root number for each shoot (Table 5).

In the comparative study of non-rooted TC shoots and those rooted in vitro the highest percentage of survival was seen for the previously rooted shoots. Of the rooted shoots in both the environment of the plastic box and mist, 97% survived (Table 6). This was significantly greater than that for the non-rooted shoots. Here also the environment was found to have no significant influence on survival.

Although a significantly lower survival percentage was seen on transplanting the TC plants as compared to the seedlings, the rates of survival were relatively high. The rates of 89% under the plastic box and 73% under mist for the in vitro grown shoots are higher than that observed in other species; 60% for blackberry (16), 50% for rose (88), and 50% for carnation (31).

In general, intermittent mist had the same effect as the plastic box environment on transplantation survival. This was similar to the report in micropropagated Dieffenbachia (78) where both mist and a plastic tent were equally effective in ensuring survival. Increased growth seen under mist in Dieffenbachia was suggested to be due to increased light levels. The increase in root weight under mist in Paulownia may also be a reflection of general increased growth under mist.

Rooting in vitro had a significant effect in improving the chances of survival, although it was not as drastic as in Vanilla planifolia (58) where none of the unrooted plants survived. Nevertheless, since

TABLE 5

EFFECT OF METHOD OF CULTURE AND ENVIRONMENT  
ON PRIMARY ROOT NUMBER OF TRANSPLANTED SHOOTS  
OF PAULOWNIA TOMENTOSA

Culture	Mean Root Number/Shoot <sup>y</sup> Environment	
	Plastic Box	Mist
Seedling	3.3	3.6
Tissue Culture	3.2	3.2
Plastic Box vs. Mist		NS <sup>z</sup>
Seedling vs. Tissue Culture		NS
Culture x Environment		NS

TABLE 6

EFFECT OF ROOTING IN VITRO ON PERCENTAGE OF SURVIVAL  
OF TRANSPLANTED SHOOTS OF PAULOWNIA TOMENTOSA

Rooting	Percentage of Survival <sup>y</sup> Environment	
	Plastic Box	Mist
Rooted	97.0	97.0
Non-rooted	89.3	95.3
Rooted vs. Non-rooted		**y
Plastic Box vs. Mist		NS
Rooting x Environment		NS

<sup>z</sup>Nonsignificant (NS), or significant at 5% (\*), 1% (\*\*), 0.1% (\*\*\*) levels.

<sup>y</sup>Mean of 6 replications, with 50 shoots/replication.

survival rates of unrooted plantlets was considerably high, elimination of in vitro rooting is recommended. This is also advised by Debergh and Maene (27) who argued against the practice of rooting in vitro from both an economical and physiological point of view. They believed that a continuous auxin regime in the rooting medium was not suitable since it hindered root elongation after root initiation. One technique suggested was pre-treatment of shoots with aqueous auxin solution in vitro followed by direct planting of shoots into an artificial planting medium that had been previously saturated with an auxin solution. This simple measure can be adopted for Paulownia. The transplanting techniques investigated appear to be satisfactory and additional precautions seem unwarranted.

#### Cold Storage

In the first method of cold storage, with shoots separated, excised, and placed in fresh media after cold storage proliferation was higher for the shoots stored at the lower temperature of 4°C (Figure 6). At this temperature, there was initially an increase in the proliferation rate from the standard of 6.1/shoot. Thereafter it declined steadily with increase in days of storage, reaching a low of 3.2 after 63 days in cold storage. The proliferation rate was lower throughout for the shoots stored at 7°C with no initial increase and a rate of 3.0 after the 63 day period (Figure 6).

The second method of storage involved excision of shoot tips and placement in fresh proliferation media prior to storage. This method

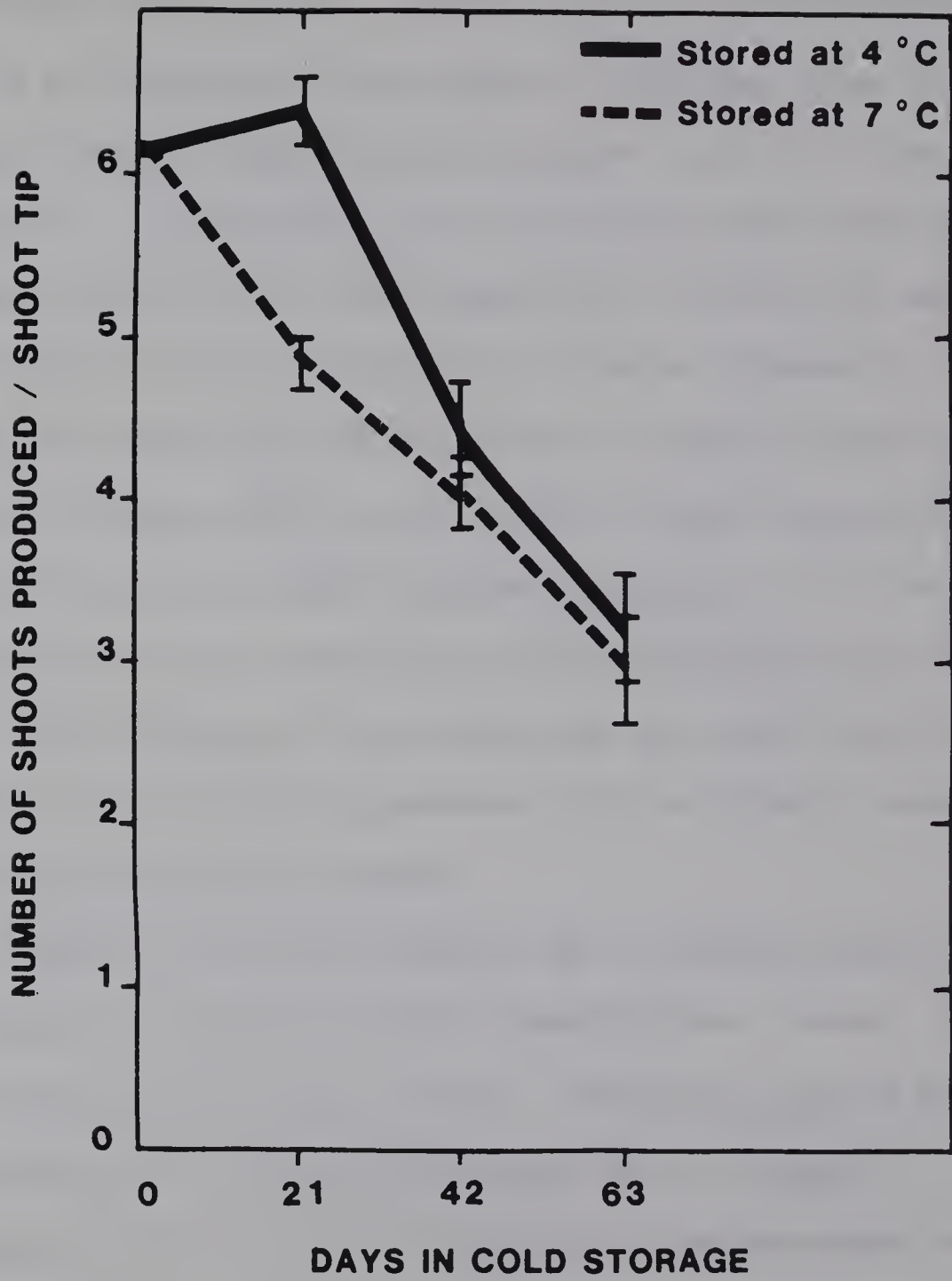


Figure 6. Shoot proliferation rate of shoots of Paulownia tomentosa after in vitro cold storage by method 1.

did not result in any clear distinction between the effects of the two different storage temperatures. A decline in the proliferation rate occurred from the beginning in both cases. Initially, after 21 days of storage, the rates were approximately the same: 3.6 at 4°C and 3.5 at 7°C (Figure 7). Subsequently, the rate declined much more rapidly for the shoots stored at the lower temperature, but after 84 days of storage the rates were not significantly different (Figure 7).

Although the rates of proliferation were in general lower for the second method, storage could be achieved for a longer period (84 days). When shoots were clumped together in storage, as in the first method, they were not in a condition for multiplication beyond 63 days of storage. The advantage of this method was that upon removal from storage, shoots with a healthy appearance could be visually selected and the excised tips placed in media.

Since a number of practical benefits can be obtained from this simple technique, it deserves further investigations. Lower temperatures might yield better results. Storage in vitro of plants such as strawberry (71), Lolium multiflorum (23), and Lotus corniculatus (105) were all done successfully at temperatures between 1-4°C. More of the shoots of Paulownia buried in media appeared to remain viable. Hence, placement of shoots horizontally deep in the media could be attempted. The decline in the proliferation rate of cold stored shoots of Paulownia may actually be a consequence of delayed proliferation. The stored shoots were kept under ambient conditions for only 21 days after removal from storage. Initial growth may have been hindered by previous cold temperatures. If shoots were

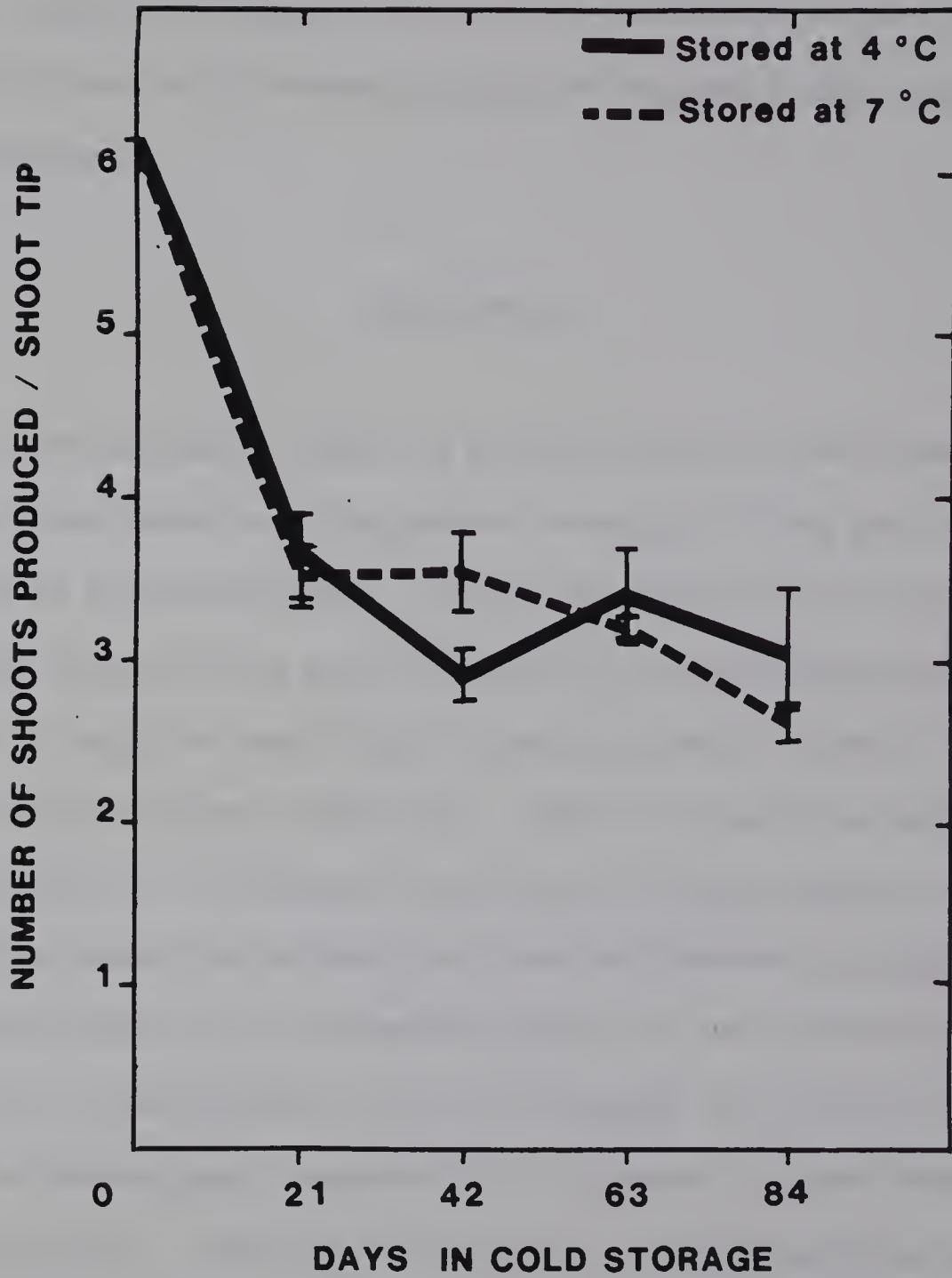


Figure 7. Shoot proliferation rate of shoots of Paulownia tomentosa after in vitro cold storage by method 2.

harvested later, the number of recovered shoots may not have decreased. Despite the apparent decline in proliferation rate with short term storage, cold storage in vitro of Paulownia shoot cultures appears promising.

### Conclusion

A conclusive statement about the variation seen in the adventitious in vitro produced shoots and the general stability of the population would appear to be contradictory. From a practical point of view, however, this situation has great potential for future exploitation. There exists a tangible possibility of getting useful variants that cannot be dismissed as mere artefacts. These can easily be separated out and propagated. Detection of aberrations in vitro would be even more beneficial since the variant could be proliferated in vitro. An illustration of this is the variegated clone that was detected and proliferated. At the standard rate of 6.1/shoots in 21 days, 1,385 shoots can be theoretically expected to be produced in three months from a single shoot. This cannot be done by using conventional methods of asexual propagation. There are now chances of obtaining a stable periclinal chimera which could be of ornamental value.

At the same time, for micropropagation and germplasm preservation of Paulownia, assumptions can be made about maintenance of quality and uniformity of the regenerated plants. There were no phenotype variants observed when shoot cultures were initiated and maintained from terminal or axillary buds. In addition, the success obtained with

transplantation of the tissue cultured shoots to the external environment and the positive indications of cold storage in vitro creates even more optimism about the prospects of Paulownia tomentosa in tissue culture. Additional studies aimed at regenerating plants from callus tissue or cell suspensions would prove to be beneficial since the induction of variation and cell selection techniques could be initiated at the cellular level.

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APPENDIX A

TABLE 7

COMPONENTS OF MURASHIGE AND SKOOG (72) BASAL MEDIUM (mg/l)

Major Salts

$\text{NH}_4\text{NO}_3$	1650.00
$\text{KNO}_3$	1900.00
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440.00
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370.00
$\text{KH}_2\text{PO}_4$	170.00

Minor Salts

$\text{H}_3\text{BO}_3$	6.20
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.80
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.60
KI	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025

Iron Source

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.80
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	37.30

(continued)

TABLE 7 (continued)

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Organics

Sucrose	30,000.00
Myo-inositol	100.00
Nicotine Acid	0.50
Thiamine HCl	0.10
Pyrodoxine HCl	0.10
Pyrodoxine HCl	0.50
Glycine	2.00

APPENDIX B

## Figure 8.

Representative electron micrographs of leaf mesophyll cells  
of: (top) green seedling of Paulownia tomentosa,  
(center) mosaic area of variegated clone 24,  
(bottom) albino area of variegated clone 24.

