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Brand, Mark Henry, Ph.D.
The Ohio State University, 1988
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MORPHOLOGICAL AND BIOCHEMICAL EVALUATION OF THE REJUVENATION OF MICROPROPAGATED BETULA SP.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

Mark Henry Brand, B.S., M.S.

****

The Ohio State University
1988

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To My Parents
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CHAPTER I

Introduction

Phase change, or maturation, occurs in a relatively short period during the early part of the life of a plant, and includes those changes characteristic of a switch from the juvenile phase to the adult phase (Brink, 1962; Wareing, 1959). The juvenile stage of a plant is considered to be the time during which a plant cannot be induced to flower, while the adult stage is the time during which a plant can flower if conditions are appropriate. If the end of the juvenile period and the appearance of the first flowers do not coincide, the intervening period is generally considered to be the transition period. Once complete, maturation is relatively stable and can be distinguished from aging, or the gradual reduction in growth and metabolism as woody perennials increase in size and complexity (Wareing, 1959).

Associated with the transition from the juvenile to adult phase are progressive changes in morphological and developmental attributes. They include bark characteristics (Olesen, 1982), tracheid width (Olesen,
1982), tracheid length (Rumball, 1963), leaf cuticular characteristics (Franich, et al., 1977), leaf shape and thickness (Rogler and Hackett, 1975), phyllotaxis (Rogler and Hackett, 1975), stem thorniness and shoot orientation (Schaffalitzky de Muckadell, 1959), branch number and branching pattern (Libby and Hood, 1976), shoot growth vigor (Goodin, 1964; Sweet and Wells, 1974), stem pigmentation (Rogler and Hackett, 1975), seasonal leaf retention (Schaffalitzky de Muckadell, 1959), cold resistance (Hood and Libby, 1980) and the ability to form adventitious roots (Schaffalitzky de Muckadell, 1959). Changes in these characteristics are species-specific and may or may not change during the period immediately preceding the adult phase. The attainment of the ability to flower is the only conclusive way to identify the end of the juvenile phase and is exhibited universally. Of the many morphological and physiological attributes associated with phase change, those of greatest practical significance include changes in shoot growth vigor, ornamental features, the ability to form adventitious roots and the ability to flower and fruit.

For plant growers, ranging from landscape propagators to orchardists, the ability to induce or maintain juvenility, or shorten its duration, would result in increased production and profits.
Orchardists and plant breeders would benefit from the ability to shorten or eliminate the juvenile phase, either to achieve earlier bearing, or to manifest mature morphological features. The length of the juvenile period is inversely related to the breeding efficiency of woody perennials and to the selection of improved cultivars (Hansche and Beres, 1980). Conversely, plant propagators desire juvenile material in order to take advantage of the ease of cutting propagation and subsequent rapid growth of the rooted plants. Selected cultivars used in horticulture today are adult phase and are, therefore, often difficult to propagate.

Manipulation of phase change in woody plants has been accomplished to some extent. The juvenile period has been reported to be shortened through environmental treatments (Aldwinckle, 1975; Brix and Portlock, 1982; Doorenbos, 1955; Visser, et al., 1976), grafting of seedlings onto mature or clonal rootstocks (Tydeman, 1961; Zimmerman, 1972), growth retarding treatments (Zimmerman, 1972) and growth regulator treatments (Chacko, et al., 1974; Pharis and Morf, 1967; Ross, et al., 1981). In many cases, the experimental conditions were poorly controlled and it is difficult to determine if the juvenile period was actually shortened or if flowering was simply promoted during the transitional
Procedures have also been developed to obtain juvenile material from adult phase plants. They include using the juvenile parts of the mature plant (Chase, 1947; Porlingus and Therios, 1976; Smith and Chin, 1980), grafting of mature scions onto juvenile rootstocks (Monselise, 1973; Stoutemyer and Britt, 1975), treatment with plant growth regulators (Borchert, 1965; Rogler and Hackett, 1975) and severe pruning, hedging, or stooling (Black, 1972; Hatcher, 1959). It is often difficult in these studies to distinguish between ontogenetically rejuvenated plants and plants which are simply reinvigorated. These conventional practices used to manipulate phase change are unreliable, demand high maintenance and are frequently impractical.

Many researchers and micropropagators have observed that rejuvenation of woody species may occur in vitro (Broome and Zimmerman, 1978; David, et al., 1978; Economou and Read, 1986; Gupta, et al., 1981; Lyrene, 1981; Mullins, et al., 1979; Sriskandarajah, et al., 1982; Takeno, et al., 1982/3; Zimmerman, 1981). The ability to study rejuvenation and phase change in vitro would offer an ideal tool for use in studying this phenomenon. The exact nature of any phase change events occurring in vitro must be determined before
studies in phase change can be conducted employing in vitro techniques. The primary objective of this research is to positively determine the nature and extent of any rejuvenation occurring in vitro, using morphological and biochemical methods.

Although mature meristems appear to be quite stable both in vivo and in vitro, recent studies indicate that their phase-related characteristics can be modified as a result of in vitro culture. The length of culture, the number of subcultures and the type of in vitro growth or proliferation involved, seem to be related to any rejuvenation processes which occur. This suggests the importance of the culture medium and culture environment in in vitro rejuvenation.

Blueberry shoot proliferating cultures initiated from mature explants have been reported to gradually produce shoots which are similar in appearance to seedlings (Lyrene, 1981). Shoots produced had smaller leaves, thinner stems, shorter internodes and exhibited enhanced rooting ability. The initial shoots formed were larger in stature, grew slowly and were probably non-juvenile, but gave rise to finer, rapidly growing juvenile-like shoots. Mature phase shoot tips of deciduous azaleas behave similarly to blueberries (Economou and Read, 1986). New shoots forming from lateral buds of original shoot tips after 10 weeks in
culture had thick, large leaves and lacked juvenile characteristics. After subsequent subcultures, shoots began to develop juvenile characteristics such as thin stems and smaller leaves. Jonathan apple showed some reversion in leaf shape with time, but the changes were not as distinct as for other species (Sriskandarajah, et al., 1982). Rejuvenation manifested itself as lobed leaves, deeper serrations and thinner leaves.

More distinct changes in morphology have been observed in shoot tip cultures of *Vitis vinifera* 'Cabernet Sauvignon' initiated from mature tissue (Mullins, et al., 1979). The adult form of grape is characterized by the presence of tendrils and distichous phyllotaxy. Serial subculturing of shoot tips gave rise to shoots with spiral (2/5) phyllotaxy and an absence of tendrils, therefore resembling seedlings. Apparently, time in culture, or the number of subcultures is critical to the induction of *in vitro* rejuvenation. Equally striking morphological differences exist between adult and juvenile *Morus*, in that adult leaves are entire, while juvenile leaves are lobed. *In vitro* regeneration of mulberry plants from mature phase axillary buds has resulted in some plantlets which have lobed leaves, indicating a more juvenile state (Patel, et al., 1983). Other plantlets had entire leaves and *in vitro* flowering was observed
under some treatments. It is unclear whether the inflorescences were preformed or developed in vitro.

Plants which have simple leaves in the juvenile phase and compound leaves in the adult phase have provided ideal systems for the observation of morphological changes indicating rejuvenation in vitro. In Rubus, which has trifoliolate mature leaves and monophyllous juvenile leaves, it was observed that mature phase shoot tips expanded a few preformed trifoliolate leaves when placed in vitro, but quickly produced only monophyllous leaves (Broome and Zimmerman, 1978). Although the production of only monophyllous leaves by shoots was stable in vitro, once rooted, shoots rapidly switched back to trifoliolate leaf production and no delay in flowering was noted. This would suggest that in vitro rejuvenation may only be a partial rejuvenation, or that only morphological characteristics are affected and not flowering ability. Maintenance of simple leaves in vitro may be due to heterotrophic nutrition. Mature shoot tips of Sambucus produce compound leaves, but when placed in vitro on sucrose and vitamin containing medium, switch to the production of simple leaves (Hrib, et al., 1980). Removal of sucrose and vitamins from the culture medium, or transferral of rooted shoots to soil, results in the subsequent production of compound
leaves. Heterotrophic nutritional conditions may, therefore, be necessary to cause or maintain *in vitro* rejuvenation, while autotrophic nutritional conditions may stabilize maturity.

A progressive improvement in the rooting of microcuttings has often been cited as an indication of *in vitro* rejuvenation. Newly initiated shoot tip cultures of Jonathan apple failed to root, but after nine subcultures, 95% rooting was achieved (Sriskandarajah, et al., 1982). Twenty-year-old mature *Eucalyptus citriodora* exhibited no rooting during early subcultures, but by the fifth and subsequent subcultures, rooting of 45 to 50% was attained (Gupta, et al., 1981). Similarly, rooting of initial *Rhododendron* shoots was poor, but after five subcultures rooting was excellent (Economou and Read, 1986). It has been intimated that the ease with which *Rhododendron* roots in culture may be due to its more juvenile physiology (Douglas, 1984). In apple, the increase in rooting ability with increasing numbers of subcultures was associated with a reduction in endogenous gibberellin and cytokinin levels (Takeno, et al., 1982/3).

Although the rooting behavior of micropropagated shoots suggests that *in vitro* rejuvenation occurs, plants propagated through micropropagation do not
always show obvious delayed maturation or flowering in the field. Micropropagated apples varied between cultivars in their precocity to flower (Webster, et al., 1985). The variety 'Greensleeves' exhibited controlled growth and flowered as early and as heavily as plants of the same variety grafted on dwarfing rootstocks. 'Golden Delicious' and 'Cox's Orange Pippin' grew too vigorously as micropropagated plants and were less fruitful than grafted plants. This suggests that the extent and stability of in vitro rejuvenation varies with the genotype. In blueberry, cuttings taken from micropropagated plants grown in vivo for three months rooted as though they were juvenile and exhibited juvenile morphology, demonstrating that rejuvenation in blueberry is stable for at least three months outside of culture (Lyrene, 1981). For other species, such as blackberry (Broome and Zimmerman, 1978) and elderberry (Hrib, et al., 1980), adult morphology is reattained quickly.

Environment may play a significant role in the modification of morphological, physiological and growth characteristics of cultured plants. Studies using Betula platyphylla var. szechuanica, comparing juvenile microcultured shoots, seedlings grown in microculture-like conditions and juvenile micropropagated plants grown in the greenhouse, showed
that seedlings grown under microculture-like conditions were intermediate in morphology and physiology to tissue grown under the other two conditions (Smith, et al., 1986). Seedlings and tissue-cultured seedlings of the Asian white birch planted in the field showed similar growth rates, but micropropagated seedlings stopped growth and exhibited fall color one month ahead of seedlings (McCown and Amos, 1979). Physiological differences other than shifts in maturity may be induced by micropropagation; however, in this study, the plant material was not clonal and the observed differences could be due to genetic differences.

Although there is considerable evidence in support of in vitro rejuvenation, evidence also exists demonstrating the stability of the mature phase. Callus cultures derived from stem tissues of mature and juvenile Hedera have characteristically different cell proliferation rates and cell sizes, which are stable for at least two years (Stoutemyer and Britt, 1965). Intrinsic differences in unorganized cells of mature and juvenile forms may therefore exist. Similar results have been seen with Castanea vulgaris, Robinia pseudoacacia and Aesculus hippocastanum (Trippi, 1963a,b). Mature and juvenile cell lines were shown to proliferate variably, dependent on the season. When mature and juvenile apical meristems were placed in
culture, stable cell proliferation rate differences were again seen for *Hedera* (Polito and Alliata, 1981). It seems, therefore, that the physiological differences between mature and juvenile cells are stable and expressed in the absence of factors associated with sub-apical organized tissue.

Stability of the mature phase has also been seen in organized tissue. Different treatments were needed to achieve optimum rooting of microcuttings obtained from adult phase and juvenile phase shoot proliferating cultures (Welander, 1983; Welander and Huntrieser, 1981), indicating that adult tissue had not rejuvenated during shoot proliferation. It is possible that shoot proliferation was not maintained long enough to facilitate rejuvenation before microcuttings were harvested.

Adventitious bud and embryo formation has been speculated to be a rejuvenating process. Adventive somatic embryogenesis from nucellar tissue of 'Cabernet Sauvignon' grape has produced plants which appear juvenile, having spiral phyllotaxy and no tendrils (Mullins and Srinivasan, 1976). The morphological changes could be due to cytological abnormalities or to the possible juvenile status of the nucellus. Brink contends that the nucellar tissue is juvenile (Brink, 1962), but this conclusion may be unwarranted based on
the occurrence of juvenile characters in plants derived adventitiously from tissue known to be mature. Callus initiated from mature tissue of 'Seyval' grape gave rise to adventitious embryos and the plantlets appeared juvenile (Krul and Worley, 1977). Mature leaf primordia fragments of grape have also been shown to give rise to adventitious shoots and plantlets with seedling characteristics (Barlass and Skene, 1980). *Hedera helix* callus initiated from mature and juvenile tissue regenerates adventitiously through different modes. Mature callus undergoes adventive embryogenesis, while juvenile callus produces adventitious buds (Banks, 1979). Both modes of adventitious regeneration give rise to juvenile plantlets.

There are a few cases where adventitious regeneration did not appear to cause rejuvenation. One case involves adventitious bud formation on callus derived from very early flowering birch seedlings which also exhibited a low propensity to root (Huhtinen, 1976). Plants derived adventitiously produced male flowers in two months and female flowers in six months on all of the plantlets. Therefore, the earliness to flower appears to be maintained through adventitious bud formation. It was noted that some vegetative buds formed on micropropagated plants, where seedlings have
only generative buds, so perhaps some rejuvenation was evident. In another instance, leaf, stem and tendril explants of Passiflora suberosa were taken from the lower and upper part of mature plants and also from juvenile plants (Scorza and Janick, 1980). All juvenile tissue and tissue from below the fifth node produced vegetative growth, but explants from the upper (mature) part of the plant flowered. When leaf disks were recultured from flowering shoots they formed vegetative shoots, so the ability to flower was lost with time in culture. Perhaps the strongest evidence supporting the notion that a mature meristem can be formed de novo is with ginseng. Embryoids which formed from root callus of mature Panax ginseng flowered in the embryoid stage while still in culture (Chang and Hsing, 1980). Because there are reports of the retention of mature qualities in tissues derived adventitiously, it is unclear whether de novo meristem formation implies rejuvenation.

If intrinsic differences do exist in the meristematic cells of mature and adult tissue, these differences might be expected to be reflected in quantitative or qualitative differences in deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or proteins. The DNA content of mature and juvenile Hedera helix cells has been evaluated by many
laboratories. Some researchers have found the levels of DNA to be higher in mature phase leaf tissue on a dry weight or cellular level (Kessler and Reches, 1977; Millikan and Ghosh, 1971). Other researchers have found that nuclei of mature bud and leaf cells contained an average of 71% more DNA than comparable juvenile tissues (Schaffner and Nagl, 1979). A majority of workers report no differences in DNA content per cell between juvenile and mature tissues of shoot apices (Wareing and Frydman, 1976), apical buds (Domoney and Timmis, 1980), apical meristems (Polito and Alliata, 1981) and stem callus (Hackett, et al., 1964).

On a dry weight basis, total, soluble, and ribosomal RNA content in mature Hedera helix leaf tissue has been reported to be less than in juvenile leaf tissue (Millikan and Ghosh, 1971). Similarly, total RNA per cell has been found to be higher in juvenile callus than in mature callus (Hackett, et al., 1964). The results of rRNA hybridization experiments with DNA prepared from mature and juvenile tissue provide no evidence for differences in rRNA redundancy between the two forms (Domoney and Timmis, 1980). In other DNA-RNA hybridization studies designed to examine RNA populations, differences in RNA species were found between mature and juvenile forms, but differences were
observed in the frequency distribution of RNA species (Rogler and Dahmus, 1974).

If differences exist in nucleic acids between mature and juvenile tissue, it follows that differences should be seen in protein composition. Isozyme analysis is commonly used to distinguish cultivars biochemically (Kuhns and Fretz, 1978a,b; Menendez and Daley, 1986; Menendez, et al., 1986). Isozyme patterns have also been used to distinguish between physiological differences in the same genotype (Scandalios, 1974). For Betula pendula callus, isozyme patterns were found to vary for growing and differentiating callus (Srivastava and Steinhauer, 1981). Electrophoretic separation of basic proteins of Hedera helix tissue cultures in the adult and juvenile form revealed some differences in protein banding (Fukasawa, 1966). Some bands were more dense in extracts from mature callus and one band was more dense in extracts from juvenile callus. Peroxidase activity has been used as a biochemical marker in globe artichoke to quantify the reacquisition of characteristics similar to those of young seedlings (Moncousin, 1982). Enzyme activities have also been used as biochemical markers of maturation status in woody plants (Trippi, 1963c). Catalase activity was found to be lower in adult tissue than in juvenile
tissue and p-phenol oxidoreductase activity increased initially with maturation, but then declined as plants aged. Amylase and peroxidase activity appear to increase with age, but variability and lack of comparability of absolute activity make interpretation difficult.
CHAPTER II

Morphological Evaluation of the In Vitro Rejuvenation of Betula sp.

Introduction.

Phase change is defined as the events which occur when a young plant (generally a woody perennial) passes from the juvenile state, when it cannot flower under any conditions, through a transitional phase, to the adult stage, when flowering can occur if the proper environmental conditions are provided (Brink, 1962). Associated with phase change in many species are progressive changes in morphological and developmental attributes (Schaffalitzky de Muckadell, 1959; Rogler and Hackett, 1975a). A majority of selected cultivars and clones used in horticulture are selected based on desirable mature-phase features. Once a plant has attained sufficient maturity to allow for proper selection, it is often difficult to obtain juvenile material of the same genotype for propagation.

It has been demonstrated that, as tree age increases, the adventitious rooting ability of shoot cuttings declines (Gardner, 1929; Stoutemyer, 1937;
Libby and Conkle, 1966). Plant propagators and nursery growers desire juvenile material in order to take advantage of the ease of cutting propagation and subsequent rapid growth of the rooted plants. Phase change is an epigenetic event, with the mature phase being relatively stable once attained. To achieve rejuvenation or obtain juvenile propagules, juvenile parts of mature plants have been used (Chase, 1947; Porlingus and Therios, 1976; Smith and Chin, 1980), mature scions have been grafted onto juvenile rootstocks (Monselise 1973; Stoutemyer and Britt, 1961; Clark and Hackett, 1980), plant growth regulators have been applied (Borchert, 1965; Rogler and Hackett, 1975a), and severe pruning, hedging or stooling have been employed (Hatcher, 1959; Black, 1972). These conventional methods of obtaining juvenile forms of selected genotypes are unreliable, demand high maintenance, and are frequently impractical. It is often difficult to distinguish between ontogenetically rejuvenated plants and plants which are simply reinvigorated by these techniques.

Recent studies indicate that the phase-related characteristics of mature meristems can be modified as a result of in vitro culture. Blueberry cultures initiated from mature explants have been shown to gradually produce shoots which are similar in
appearance to seedlings (Lyrene, 1981). Similar development of juvenile morphological characteristics in vitro has been reported for deciduous azaleas (Economou and Read, 1986) and Jonathan apple (Sriskandarajah, et al., 1982), but the differences in juvenile and mature growth are subtle. Species which show more distinct morphological differences between the mature and juvenile forms, such as grape (Mullins, et al., 1979), mulberry (Patel, et al., 1983), elderberry (Hrib, et al., 1980), and raspberry (Broome and Zimmerman, 1978), have also shown morphological reversion to the juvenile state in vitro.

Struve and Lineberger (1988) found that microcuttings of paper birch (Betula papyrifera), harvested from shoot proliferating cultures (initiated from flowering trees) rooted as well or better than cuttings taken from seedlings. The rooting response of micropropagated plants which reached the 12-13 node stage was shown to be intermediate between that of seedlings of similar size and mature-phase cuttings. This evidence indicates that in vitro culture of paper birch may induce a partial reversion to a more juvenile state. Work with early flowering forms of birch suggests that rejuvenation may not occur in tissue cultures of Betula sp. (Huhtinen, 1976). The earliness to flower was maintained in all plants derived
adventitiously. Some vegetative buds formed at the shoot terminals, where seedlings typically form generative buds; this may be an indication of rejuvenation, in spite of the early flowering. McCown and Amos (1979) grew seedlings and tissue-cultured seedlings of the Asian white birch in the field. The two types of plants showed similar growth rates, but micropropagated seedlings stopped growth and exhibited fall color one month ahead of seedlings. Physiological differences other than shifts in maturity may, therefore, be induced by micropropagation. The plant material used in this study was not clonal, and the observed differences could be due to genetic differences.

In this study, some morphological features and developmental aspects which have been shown to change with maturation were evaluated in variously-propagated clonal paper birch, to gain evidence to support or refute the notion of in vitro rejuvenation in this species.

Materials and Methods.

All plant material was originally obtained from 15-year-old, grafted seed orchard clones at the Mount
Alto Pennsylvania State Forestry Nursery. An outline showing the various types of plants used in this study, and their derivation, is provided in Figure 1. Three clones were used in this study; two Betula papyrifera clones (231 and 273) and one Betula pubescens x papyrifera hybrid (247). These clones were selected based on their various in vitro shoot proliferation rates and tendencies to form shoots adventitiously, since evidence exists that adventitious events may induce rejuvenation (Barlass and Skene, 1980; Krul and Worley, 1977).

Tissue cultures were initiated from shoot tips taken from flowering branches, and shoot proliferating cultures were maintained on Woody Plant (WP) medium (Lloyd and McCown, 1980) containing 2.5 mg l⁻¹ benzyladenine (BA). Other culture conditions were as described by Struve and Lineberger (1988). Shoot proliferating cultures had been maintained for 2 to 3 years when 2 to 3 cm microcuttings were harvested and rooted under non-sterile conditions, in plastic-covered aluminum trays containing a 1:1 mixture of finely screened peat moss and vermiculite. Microcuttings did not receive an auxin treatment prior to a four week rooting period under culture room conditions.

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¹Seed and scion wood collected by J. Winieski.
Figure 1. Diagram outlining the derivation of the various plant types used in this study.

Evaluations were conducted on 100 plants of each clone for seedlings and tissue-cultured plants. A mean number of 48 grafted plants and 14 cutting-propagated plants was evaluated for each clone.
FIGURE 1.
Acclimation of rooted microcuttings was achieved by repotting the plantlets into cell packs containing Metro Mix 350\(^2\), and placing them in intermittent mist and shade for seven days, followed by seven days in a shaded greenhouse.

Open pollinated seeds of the three clones were collected directly from the original trees in Pennsylvania, and were stored dry at 4\(^\circ\)C until needed. Seeds were broadcast on Metro Mix 350 and placed in intermittent mist, in conditions identical to those used to acclimate microcuttings. Seeds were sown at the same time that microcuttings were stuck to root, in order to yield seedlings and tissue-cultured plantlets of similar size and developmental status. Seedlings were acclimated identically to tissue-cultured plantlets. Resulting seedlings were used to represent the "true juvenile" state, or were used as juvenile rootstocks, onto which mature scions were grafted.

Grafting of mature scions onto juvenile stocks has been demonstrated to result in possible rejuvenation (Monselise, 1973; Stoutemyer and Britt, 1961). Therefore, grafted plants were included for comparison to "true adult" plants. Six-month-old, half-sib seedlings were used as rootstocks, and mature-phase buds from flowering plants were chip-bud grafted onto

\(^{2}\)W. R. Grace, Cambridge, MA.
them 4 inches above the soil line. Budding was performed in February; in each case, the appropriate scion genotype was placed on its half-sib juvenile counterpart. Once a scion initiated growth, the top of the rootstock was removed, and the scion was trained to a single leader.

To represent the "true adult" state, two-year-old cutting propagated plants were used. The plants were dug from field plantings at the Lane Avenue Horticulture Farm (Columbus, OH) in November, and were stored bareroot at 4°C until February. All of the cutting-propagated plants were flowering. Plants were potted and were allowed to initiate a spring growth flush.

All four types of plants (seedlings, micropropagated plants, grafted plants, and cutting-propagated plants) were initiated or allowed to begin active growth at similar, but not necessarily identical times. This was done in an attempt to match the length and vigor of the current growth flush on each type of plant, and to synchronize the physiological states which existed in the plants.

Grafted plants, cutting-propagated plants, and older seedlings and tissue-cultured plants were grown on a pine bark:peat moss:sand (3:1:1) medium, supplemented with 0.12 kg dolomite per m³. Plants
were fertilized weekly with 200 ppm N from a water-soluble fertilizer (20:20:20, N:P:K), and received periodic applications of soluble trace element mix (STEM). Plants were grown in plastic containers and the container size was adjusted up as required by plant growth. Diseases and insects were controlled by timely foliar sprays.

Natural daylength was supplemented with 30-60 μE m⁻² s⁻¹ photosynthetically active radiation (PAR) supplied by high-intensity sodium vapor lamps, to provide a continuous dawn-to-dark, 16 hr photoperiod during the course of the study. Greenhouse temperatures averaged 25°C/18°C (day/night).

Morphological evaluations were conducted at 1, 4, and 8 months post-acclimation for seedlings and tissue-cultured plantlets; at each collection time, data were also taken from grafted and cutting-propagated plants. For each data collection time, the seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants that were evaluated had grown at the same time, under the same greenhouse and seasonal conditions. Sufficient numbers of plants were grown to allow for destructive harvest when necessary, without influencing subsequent evaluations.

Plants were inspected for the presence or absence of flowers, white bark on the main trunk, and
anthocyanin development in growing shoot tips. The length of the fifth and sixth internodes from the shoot apex and the stem diameter at the fifth internode were measured. As an attempt to numerically evaluate leaf shape for use in comparisons between plant types, the length and width of the fifth leaf from the apex were measured at the longest and widest points. A length-to-width ratio was calculated. In general, internodes and leaves at or between the fifth and sixth nodes were the youngest, fully-expanded organs of their type on the plant. To measure pubescence, the number of hairs per cm of stem was determined for the fifth internode, using a Zeiss dissecting microscope with a 2.0X objective. Low counts were actual counts of the total hairs per cm of stem, while high counts were estimated from counts on two mm sections of tissue. One way analysis of variance was conducted using SPSS PC+, version 1.1.

Results.
All cutting-propagated plants flowered, and both male and female flowers were present. Seedlings and tissue-cultured plants did not flower, even after eight months post-acclimation. Grafted plants of clones 231 and 247 produced some flowers by the four- and
eight-month periods (Table 1). The majority of flowers on grafted plants were male.

White bark was present on all cutting-propagated plants (Table 2). Grafted plants of clone 247, observed at the eight-month period, were the only other plants which developed white bark during the course of the study.

Anthocyanin development was never observed in the shoot tips of cutting-propagated or grafted plants at any of the observation times (Table 3). Seedlings exhibited the greatest amount of anthocyanin development, but tissue-cultured plants of clones 247 and 273 showed similar levels of anthocyanin development. Only 10% of clone 231 tissue-cultured plants developed anthocyanin at one month post-acclimation, and by four months, no visible anthocyanin was present. With increasing time after acclimation, fewer seedlings and tissue-cultured plants showed anthocyanin development. Tissue-cultured plants tended to lose the ability to produce anthocyanins in growing shoot tips at a faster rate than seedlings.

At any given evaluation time, stems of grafted and cutting-propagated plants were less pubescent than those of seedlings and tissue-cultured plants, with the exception of micropropagated 231 plants, which did
Table 1. Incidence of flowering (%) for three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation.

<table>
<thead>
<tr>
<th>Incidence of Flowering (% of total)</th>
<th>Clone 231</th>
<th>247</th>
<th>273</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 4 8</td>
<td>1 4 8</td>
<td>1 4 8</td>
</tr>
<tr>
<td>S</td>
<td>0a 0a 0a</td>
<td>0a 0a 0a</td>
<td>0a 0a 0a</td>
</tr>
<tr>
<td>TC</td>
<td>0a 0a 0a</td>
<td>0a 0a 0a</td>
<td>0a 0a 0a</td>
</tr>
<tr>
<td>G</td>
<td>0a 0a 8b</td>
<td>0a 53b 73b</td>
<td>0a 0a 0a</td>
</tr>
<tr>
<td>C</td>
<td>100b 100b 100c</td>
<td>100b 100c 100c</td>
<td>100b 100b 100b</td>
</tr>
</tbody>
</table>

*Means within columns, followed by the same letter, are not statistically different at the $\alpha = 0.05$ level, using Tukey's HSD procedure for mean separation.*
Table 2. Incidence of white bark (%) for three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation.

<table>
<thead>
<tr>
<th>Incidence of White Bark (% of total)$^k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 231</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
<th>0a</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>G</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>48b</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>C</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
<td>100b</td>
</tr>
</tbody>
</table>

$k$Means within columns, followed by the same letter, are not statistically different at the $\alpha = 0.05$ level, using Tukey's HSD procedure for mean separation.
Table 3. Incidence of anthocyanin (%) for three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation.

<table>
<thead>
<tr>
<th>Incidence of Anthocyanin (% of total)¹</th>
<th>Clone 231</th>
<th>247</th>
<th>273</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  4  8</td>
<td>1  4  8</td>
<td>1  4  8</td>
</tr>
<tr>
<td>S</td>
<td>57b  35b  8b</td>
<td>96b  94b  38c</td>
<td>100b  93b  50b</td>
</tr>
<tr>
<td>TC</td>
<td>10a  0a  0a</td>
<td>94b  84b  33b</td>
<td>100b  84b  33b</td>
</tr>
<tr>
<td>G</td>
<td>0a  0a  0a</td>
<td>0a  0a  0a</td>
<td>0a  0a  0a</td>
</tr>
<tr>
<td>C</td>
<td>0a  0a  0a</td>
<td>0a  0a  0a</td>
<td>0a  0a  0a</td>
</tr>
</tbody>
</table>

¹Means within columns, followed by the same letter, are not statistically different at the $\alpha = 0.05$ level, using Tukey's HSD procedure for mean separation.
not exhibit the degree of pubescence seen in similar plants of clones 247 and 273 (Table 4, Plates I-III). In addition to being less pubescent, stems of mature-phase plants were glabrous and scaly, and displayed numerous glandular structures. Seedlings were usually more pubescent than tissue-cultured plants, and tissue-cultured plants lost this quality faster than seedlings. Similar patterns in levels of pubescence could be seen on other vegetative plant parts, such as petioles and leaves (Plate IV).

Stem diameters of seedlings and tissue-cultured plants at the one-month stage were approximately one-quarter as large as those of their mature-phase counterparts (Fig. 2). By the four-month stage, seedlings and tissue-cultured plants had attained stem diameters which were two-thirds to three-quarters as large as the stem diameters of grafted and cutting-propagated plants. After eight months of growth in the greenhouse, stem diameters of all four types of plants were nearly equal.

Patterns for internode length were nearly identical for both the fifth and sixth internodes from the apex (Figs. 3 and 4). Internodes were longest on grafted and cutting-propagated plants. At one month post-acclimation, seedlings and tissue-cultured plants had internode lengths less than one-eighth as long as
Table 4. Pubescence on stems of three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation.

<table>
<thead>
<tr>
<th>Number of Stem Hairs per cm of Stem</th>
<th>Clone 231</th>
<th>247</th>
<th>273</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 4 8</td>
<td>1 4 8</td>
<td>1 4 8</td>
<td></td>
</tr>
</tbody>
</table>

S 3463b 2638c 570b 7098b 5023c 1185c 6184c 5137c 827c
TC 465a 201b 175a 1003b 3908b 755b 5565b 3328b 473b
G N/A 141a 145a N/A 83a 83a N/A 132a 136a
C 580a 140a 138a 681a 79a 79a N/A 568a 134a 133a

*Means within columns, followed by the same letter, are not statistically different at the α = 0.05 level, using Tukey's HSD procedure for mean separation. A maximum of 15 shoots per clone x plant type combination was evaluated.*
Plate I. Pubescence on stems of *Betula* sp., clone 231. From top to bottom: seedling, tissue-cultured plant, grafted plant, cutting-propagated plant.
PLATE I.
Plate II. Pubescence on stems of *Betula* sp., clone 247. From top to bottom: seedling, tissue-cultured plant, grafted plant, cutting-propagated plant.
Plate III. Pubescence on stems of *Betula* sp., clone 273. From top to bottom: seedling, tissue-cultured plant, grafted plant, cutting-propagated plant.
Plate IV. Pubescence on leaves of *Betula* sp., clone 247. Abaxial side of leaf is shown. Leaf piece on top is juvenile; leaf piece on bottom is from a cutting-propagated plant (mature).
PLATE IV.
Figure 2. Stem diameter of three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation. Lines above bars represent the standard error of the mean.
FIGURE 2.

Stem Diameter (cm)

0.50
0.40
0.30
0.20
0.10
0.00

Time Post-acclimation/Post-germination

1 Month 4 Months 8 Months
Figure 3. Internode length in three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation. Lines above bars represent the standard error of the mean. Internode measured was the fifth internode from the shoot apex.
FIGURE 3.
Figure 4. Internode length in three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature *Betula* sp. following 1, 4 and 8 months post-acclimation. Lines above bars represent the standard error of the mean. Internode measured was the sixth internode from the shoot apex.
**FIGURE 4.**

Graph showing internode length (cm) over time post-acclimation/post-germination for different groups:
- Seedling
- TC
- Grafted Cutting

Data points include:
- 231
- 247
- 273
those found on mature-phase plants. With time, internode lengths of seedlings and micropropagated plants increased, but did not equal internode lengths of grafted and cutting-propagated plants, even at the eight-month stage. One exception was eight-month-old tissue-cultured plants of clone 231, which had internode lengths that were indistinguishable from internode lengths of grafted and cutting-propagated plants. Between one and four months post-acclimation, internode lengths of tissue-cultured plants increased at a slightly faster rate than did internode lengths of seedlings. Change in internode length in tissue-cultured plants, between one and eight months, is clearly a progressive process (Plate V). Initially, internode lengths resembled those seen in seedlings but, after passing through a transition stage, characterized by intermediate length internodes, tissue-cultured plants produced longer, more "adult-like" internodes.

Great differences in leaf shape (Plate VI) and size existed between mature and juvenile forms of a particular clone. One month of growth in the greenhouse produced seedlings and tissue-cultured plants with much smaller leaves than either grafted or cutting propagated plants (Figs. 5 and 6). Leaf length and leaf width of seedling and tissue-cultured plants
Plate V. Nodal sections of a tissue-cultured plant, demonstrating the transition from juvenile to mature internode length. Section at the lower right represents the most basal (i.e., the most juvenile) node; section at the upper left represents the shoot apex (i.e., the most mature node).
Plate VI. Dimorphism in leaf shape between juvenile (top) and mature (bottom) specimens of *Betula* sp.
Leaves were taken from clone 247.
Figure 5. Leaf length in three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation. Lines above bars represent the standard error of the mean.
FIGURE 5.
Figure 6. Leaf width in three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation. Lines above bars represent the standard error of the mean.
FIGURE 6.

Leaf Width (cm)

- Seedling
- Grafted
- TC
- Cutting

Time Post-acclimation/Post-germination

1 Month 4 Months 8 Months

231

247

273
increased by four months, but leaves were still smaller than leaves found on grafted or true mature forms. Most differences in leaf size and shape were no longer present after eight months growth in the greenhouse.

Leaf length to width ratios were calculated for each clone at each observation time, but did not adequately represent the leaf shapes characteristic of each plant type at any given time (Fig. 7). Observations of leaf shape suggested that leaves from mature plants were narrower than leaves from juvenile plants and had rounded or acute leaf bases, as compared to cordate leaf bases seen on juvenile leaves (Plates VII-IX). Leaves from tissue-cultured plants were similar to juvenile leaves in shape and leaf base configuration. Clonal differences in leaf shape existed, where the leaf bases of leaves from grafted and cutting-propagated plants of clone 273 were cordate, while those of clones 231 and 247 were acute to rounded. As seedling and tissue-cultured plants aged, the characteristically juvenile shape of their leaves and leaf bases began to resemble those seen on mature grafted and cutting-propagated plants (Plate X). Leaf shape and leaf base characteristics of tissue-cultured plants generally became mature at an earlier stage than seedlings. Most micropropagated plants appeared to pass through a transitional phase
Figure 7. Ratio of leaf length to leaf width in three clones of seedling (S), tissue-cultured (TC), mature scion/seedling rootstock (G), and cutting-propagated mature Betula sp. following 1, 4 and 8 months post-acclimation. Lines above bars represent the standard error of the mean.
FIGURE 7.
Plate VII. Leaves from Betula sp., clone 231, four months post-acclimation. Clockwise from upper right: seedling, tissue-cultured plant, grafted plant, cutting-propagated plant.
PLATE VII.
Plate VIII. Leaves from *Betula* sp., clone 247, four months post-acclimation. Clockwise from upper right: seedling, tissue-cultured plant, grafted plant, cutting-propagated plant.
PLATE VIII.
Plate IX. Leaves from Betula sp., clone 273, four months post-acclimation. Counterclockwise from upper left: seedling, tissue-cultured plant, grafted plant, cutting-propagated plant.
Plate X. Leaves from a tissue-cultured plant, demonstrating the transition from juvenile to mature leaf shape. Leaf at lower right was taken from the base of the plant (i.e., the most juvenile part of the plant); leaf at the upper left was taken from near the shoot apex (i.e., the most mature part of the plant). Note gradual change of leaf base from cordate to more acute as one approaches the shoot apex.
between six and eight months post-acclimation, after which more mature morphology was exhibited. Only a small number of seedlings exhibited signs of significant maturation by eight months after acclimation.

Although grafted plants were nearly identical to cutting-propagated mature plants at the four and eight month observation times, some changes in morphology were apparent in the first two months of scion growth. The first five to six nodes which emerged from the chip bud exhibited adult morphology (Plate XI). The following ten to twelve nodes displayed morphology intermediate between that seen on true juvenile and true mature plants. All nodes which formed subsequent to the twelfth node displayed mature morphology. Unfortunately, numerical data were not taken at one month for many morphological features on grafted plants. Grafted plants also formed many leaves which resembled those seen on transitional tissue-cultured plants, characterized by having two prominent serrations toward the base of the leaf.

Discussion.

Seedlings and mature forms of clonal birch plants exhibit easily distinguishable morphological,
Plate XI. Initial growth from a chip-budded *Betula* sp. Scion was taken from a mature plant, and grafted onto a seedling rootstock. Note absence of pubescence on the scion, and the angular leaf bases characteristic of mature morphology.
physiological and anatomical differences. Differences in leaf and stem characteristics, bark color and texture, pigment formation and flowering are similar to changes observed for other woody species, such as *Hedera helix* (Wareing and Frydman, 1976). No particular morphological character appears to be rigidly associated with flowering in birch, and the different characteristics do not necessarily develop or change simultaneously. In *Hedera*, reversion to the juvenile growth habit can be induced by applications of gibberellic acid (GA$_3$), but reversion of individual morphological characters are observed at different GA$_3$ doses (Rogler and Hackett, 1975a). Reversion from mature to juvenile characteristics, facilitated by *in vitro* culture, appears to occur in birch. Recently acclimated, micropropagated birch plants were characterized by many juvenile features, including thin stems, small leaves, short internodes, anthocyanin development, altered leaf shape, dense pubescence and absence of white bark or flowers, giving them the appearance of seedlings. Lyrene (1981) found that tissue-cultured blueberry shoots, initiated from mature tissue, possessed thin stems with short internodes and small leaves, indicating possible rejuvenation *in vitro*. Changes in leaf shape, from the mature form to the juvenile form, have been reported
for tissue-cultured plants of Morus (Patel, et al., 1983), Rubus (Broome and Zimmerman, 1978), and Sambucus (Hrib, et al., 1980).

Tissue culture may not always induce or maintain juvenility. McKeand (1985) found that tissue-cultured plants of loblolly pine, initiated from embryonic tissues, exhibited morphology similar to mature trees. Stability of the mature phase has also been demonstrated with Hedera stem callus, where callus initiated from mature and juvenile stems had characteristically different proliferation rates that were stable for at least two years (Stoutemyer and Britt, 1965).

Walnut cultures from mature tissue change morphology and growth characteristics after 3 to 4 months of culture, producing fine-featured leaves and exhibiting greater tractability in culture (McGranahan, et al., 1988). Although such a shift appears to be a reversal to the juvenile phase, a true juvenile state probably does not exist, since plantlets flowered after 10 to 24 months out of culture. Tissue-cultured birch plantlets regained mature morphology faster than seedlings for features including anthocyanin pigmentation, pubescence, internode length, and leaf shape.

Flowering ability and the development of white bark were not regained by tissue-cultured birch during the
course of this study, indicating that these features are the first to be lost during rejuvenation and the last to be regained with maturation. Flowering capacity in *Hedera helix* is lost with very low doses of GA$_3$, when other mature morphological features are retained (Rogler and Hackett, 1975a).

Partial rejuvenation through grafting mature birch scions onto seedling rootstocks is transient and occurs soon after new scion growth is initiated. The transient nature of this rejuvenation was demonstrated by flowering in four- and eight-month-old grafted plants and the poorly developed juvenile morphology in young grafted plants. Grafting-induced rejuvenation has been successful in *Hedera* (Stoutemyer and Britt, 1961), but treatments to insure translocation across the graft union are important (Clark and Hackett, 1980). In birch, rejuvenation by grafting may have resulted by the use of chip-budding, where only a small section of mature wood is employed.

The cause of juvenile reversion in shoot proliferating cultures of *Betula* is unknown. Adventitious shoot or meristem formation may lead to rejuvenation *in vitro* (Lyrene, 1981; Banks, 1979). The adventitious event can be in the form of shoot regeneration (Barlass and Skene, 1980) or somatic embryogenesis (Mullins and Srinivasan, 1976). Some
evidence exists that adventitious events are not necessarily rejuvenating. Flowering shoots formed adventitiously on mature explants of *Passiflora suberosa*, but leaf disks recultured from flowering shoots formed only vegetative shoots (Scorza and Janick, 1980). Flowering has also occurred *in vitro* on somatic embryos of *Panax ginseng* derived from root callus of mature plants (Chang and Hsing, 1980).

Shoot proliferating cultures of clone 247 produce a majority of shoots through adventitious formation, while clone 273 produces approximately equal numbers of axillary and adventitious shoots. Clone 231 proliferates more slowly than clones 247 and 273, and most shoots arise from release of axillary buds. Shoot production through axillary release in clone 231 may not stimulate dramatic rejuvenation in this clone, and may explain the less obvious rejuvenation morphology. Tissue-cultured plants of clone 231 generally had less anthocyanin development and less pubescence than their 247 and 273 counterparts. Micropropagated plants of clone 231 were also the only tissue-cultured plants to develop internode lengths equivalent to mature plants during the eight-month study. Less dramatic rejuvenation in clone 231 may be due only to clonal differences, since axillary shoot proliferation may also result in rejuvenation. In *Vitis vinifera*,

axillary shoot proliferation resulted in some shoots with juvenile appearance, but serial subculturing was required (Mullins, et al., 1979).

These changes in morphology indicate that rejuvenation occurs during in vitro culture of Betula. The rejuvenation is likely to be only partial, and levels of juvenility comparable to seedlings are probably not attainable through standard shoot multiplication procedures. Biochemical evaluation of true juvenile and mature plants, as well as putative tissue culture revertants, will be necessary to more thoroughly characterize the nature and extent of in vitro rejuvenation.
List of References.


Mullins, M. G., Y. Nair, and P. Sampet. 1979. Rejuvenation in vitro: Induction of juvenile


CHAPTER III

Biochemical Evaluation of the In Vitro Rejuvenation of Betula sp. Through the Use of Denatured Protein and Isozyme Profiles

Introduction.

Alterations in morphology and anatomy associated with phase change have been used as markers of maturity or juvenility in woody plants (Rogler and Hackett, 1975a). Since the morphological changes are progressive, they can even be used as indicators of the relative level of physiological maturity of given tissue. Rejuvenation in vitro has been proposed to occur, based on changes in morphology (Lyrene, 1981; Patel, et al., 1983; Mullins, et al., 1979) and adventitious rooting ability (Gupta, et al., 1981; Economou and Read, 1986; Sriskandarajah, et al., 1982).

In birch, in vitro culture increased the adventitious rooting of stem cuttings (Struve and Lineberger, 1988). Micropropagated Betula plants also exhibit morphology similar to that in seedling birch, and unlike that in flowering plants (see Chapter.
II). This evidence supports the idea that rejuvenation does occur in vitro. Other work, with early flowering birch, showed that flowering was not delayed by tissue culture, but there were other morphological indications of rejuvenation (Huhtinen, 1976).

Changes in morphology, although frequently adequate indicators of phase change, may not always be sufficient to accurately determine the level of maturity of a given plant tissue. Not all plant species exhibit clear dimorphism between phases. Additionally, individual features may not change at parallel rates when a plant changes phase. Hackett (1983) states that determination of a biochemical or cytological marker(s) which is an indicator of the level of maturity is one of the more important areas of research necessary to facilitate better understanding of phase change. The discrete biochemical changes associated with phase change have not been determined. Exogenously applied GAs to stimulated rejuvenation in mature Hedera helix, and ABA applications counteracted this rejuvenation (Rogler and Hackett, 1975a,b). Unfortunately, endogenous hormone levels do not correlate with these findings (Hillman, et al., 1975).

Examination of DNA and RNA content in juvenile and mature tissues has, in some instances, shown differences between the two types of tissue (Millikan
and Ghosh, 1971; Kessler and Reches, 1977), but consistent trends are elusive (Domoney and Timmis, 1980).

Electrophoretic separation of proteins and isozyme analysis have been used to distinguish between cultivars (Kuhns and Fretz, 1978b; Menendez, et al., 1986) and between physiological states (Scandalios, 1974). In Betula pendula, isozyme patterns varied between growing and differentiating callus (Srivastava and Steinhauer, 1981). Some evidence exists which suggests that protein banding patterns (Fukasawa, 1966) and enzyme activities (Moncousin, 1982; Trippi, 1963c) can be used to distinguish between mature and juvenile tissue.

In this study, electrophoretic separation of total denatured protein and isozymes of peroxidase and esterase was employed to establish profiles characteristic of mature and juvenile birch. Comparison of protein profiles of micropropagated birch at various times post-acclimation was used to evaluate the extent of rejuvenation occurring in vitro in this species. Particular attention was paid to proteins that might have use in determining the level of juvenility existing in given trees (i.e., "juvenility factors").
Materials and Methods.

Plant material. Seedlings, tissue-cultured plants, grafted plants and cutting-propagated plants used in protein analysis were obtained, cultured and grown as outlined in Chapter II. Tissue was harvested at one, four and eight months post-acclimation, from all four types of plants. At the one- and four-month collection times, 2 to 3 cm shoot apices were collected. At the eight-month collection time, summer dormant buds were harvested from the twelfth to seventeenth nodes down from the growing tip. All tissue was collected in the morning and was immediately frozen in liquid N. At each collection time, tissue from each experimental group was kept separate, and consisted of the pooled tissue from all plants within a given clone x plant type combination. Tissue that was not immediately extracted was stored in polyethylene bags for 2 to 4 weeks at -42°+3°C, until extracted.

Protein extraction and sample preparation. One gram of tissue was ground to a fine powder in liquid N in a precooled mortar. The powder was transferred to a second precooled mortar containing 4 ml of cold (4°C) extraction solution and 0.3 g of polyvinyl-polypyrrolidone (PVPP). During the entire homogenization process, mortars were kept on ice. The contents of the mortar were homogenized for 3 min by hand with a
pestle, and samples for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were homogenized for one additional min. Native protein samples received only the initial 3 min homogenization. Extracts were placed in precooled 1.5 ml microfuge tubes, and were centrifuged at 12,000XG for 10 min at 4°C. The supernatants were removed to 1.5 ml microfuge tubes on ice. Samples not prepared for immediate electrophoresis were frozen in liquid N and stored for up to 48 h at -42°+3°C, without detectable changes in protein composition.

The SDS-protein extraction solution consisted of 0.1M Tris-HCl buffer (pH 8.3), containing 6M urea, 5mM phenylmethyl sulfonyl fluoride (PMSf) and 2% B-mercaptoethanol. The extraction solution for native proteins was more complex, and contained the following: 50 mM Tris (pH 8.3 with HCl); 1 mM disodium EDTA; 5 mM PMSf; 25 mM dithiothreitol (DTT); 3 mM sodium bisulfite; 5 mM ascorbic acid; 6 mM diethyl-dithiocarbamate; 0.1% B-mercaptoethanol; and 0.1% Triton X-100.

Samples to be used for SDS-PAGE were diluted in half with a 2X sample buffer solution, containing the following: 20% glycerol; 0.1% bromophenol blue; 0.14M SDS; 25% 4X stacking gel buffer; and 10% B-mercaptoethanol. The samples were then placed in a boiling
water bath for 5 min prior to being loaded on a gel. Native protein samples had 2 ul of 1% bromophenol blue solution and 50 ul of glycerol added to each 0.5 ml aliquot of extract. Samples were vortexed and loaded onto gels. At all times, native protein samples were kept cold.

**Total protein estimation.** Protein estimations were determined in a manner similar to that of Bradford (1976). Fifty ul of extract was combined with 2.5 ml of BioRad Protein Assay dye reagent in a test tube, and vortexed gently. The dye was allowed to bind to protein for 10 min, and the absorbence of the solution was measured at 595 nm using a Shimadzu spectrophotometer. Unknown protein concentrations were determined by comparison of absorptions to a standard curve prepared with bovine serum albumin.

**Polyacrylamide electrophoresis.** Polyacrylamide slab gels in a discontinuous system were run on a Hoefer Model SE 600 vertical slab gel unit attached to a BioRad Model 500/200 power supply. For SDS-PAGE, 5-15% acrylamide linear gradient gels were used, and 5-10% acrylamide linear gradient gels were used for native PAGE. Linear gradients were poured with a Hoefer SG50 side outlet linear gradient maker. All gels were 1.5 mm thick, and gradients were stabilized by additions of 75% sucrose solution to the
unpolymerized gel mixture containing the greater percentage of acrylamide. A 4.5% acrylamide stacking gel was poured on top of each resolving gel.

Resolving gels were buffered at pH 8.8 with 0.375 M Tris-HCl, and the stacking gels were buffered at pH 6.8 with 0.125 M Tris-HCl. The acrylamide:bis stock which was used supplied the monomer and cross-linker in a 30:0.8 ratio. Gels were polymerized with ammonium persulfate and TEMED. The tank buffer was 25 mM Tris-0.192 M glycine solution (pH 8.3). The tank buffer, stacking gel buffer, and resolving gel buffer used for SDS-PAGE all contained 3.5 mM SDS.

Gels were run from the cathode to the anode at a constant current of 20 mA per gel (native) or 30 mA per gel (SDS), for 4 h, 45 min. For SDS-PAGE, the lower tank chamber was filled with buffer to cover the resolving gel and the buffer was circulated with a magnetic stir bar to dissipate heat and maintain ambient room temperature in the gel and buffer. Precooled tank buffer was used with native gels and cold water was circulated through the heat exchanger to maintain cold temperatures and prevent denaturation of protein. Dalton Mark VII-L molecular weight markers (Sigma Chemical Co.) were run on each SDS gel.
**Protein and enzyme visualization.** Denatured gels were stained overnight in covered plastic trays in a 0.125% Coomassie Brilliant Blue R-250 solution containing 50% methanol and 10% acetic acid. Gels were destained in a mixture of 50% methanol and 10% acetic acid for 60 to 90 min, followed by additional destaining in a 5% methanol and 7% acetic acid solution. Gels were rocked gently during staining and destaining to insure uniformity in staining across the entire gel. SDS gels were fixed in 7% acetic acid after destaining.

Esterase activity was visualized by incubating the gels in a mixture of 75 mg Fast blue RR salt and 30 mg α-naphthyl acetate in 2 ml of acetone, made up to 100 ml with 0.05 M phosphate buffer (pH 7.5). The solution used for peroxidase staining contained 60 mg 4-chloro-l-naphthol in 20 ml methanol, 60 ul hydrogen peroxide, and 1.3 M sodium chloride, brought up to 100 ml with 0.01 M phosphate buffer. Native gels were incubated in the appropriate staining solution at 25°C for 2 h (peroxidase) or 3 h (esterase), and were then washed in distilled water to remove excess stain.

Native gels and SDS gels were photographed, and the intensity of individual bands was determined for SDS gels using a BioMed Instruments laser densitometer, scanning at 280 nm.
Results.

Differences in banding patterns between juvenile, mature and putative rejuvenated plants could be seen in both SDS polyacrylamide gels and enzyme-stained native polyacrylamide gels. Banding patterns changed in both native and denatured gels as time post-acclimation changed. Particular bands or regions of bands have been labeled on photographs and densitometer scans of gels to facilitate comparisons of the protein profiles present in each plant type at each time. In SDS gels, the bands believed to represent the large subunit of ribulose-1,5-bisphosphate (RuBP) carboxylase (in the range of 50 to 58 kd [Werner-Washburne, et al., 1983]) and the small subunit of RuBP carboxylase (in the range of 12 to 18 kd [Block, et al., 1983]) have been labeled 1 and 2, respectively.

In SDS-PAGE, the greatest differences in peptide banding patterns existed in plant tissue collected one month post-acclimation. For clones 231 and 247, protein banding patterns were identical for seedlings and tissue-cultured plants at this time, but were different from those of cutting-propagated plants (Plates XII, XIII; Figures 8, 9). Patterns for clone 273 were similar to those of 231 and 247, but the differences were not as pronounced (Plate XIV; Fig. 10). Shoot proliferating cultures of all three
Plate XII. Denatured protein profiles of one-month-old tissue of *Betula*, clone 231. Lanes 1 to 4 show profiles of tissue from shoot proliferating cultures, seedlings, tissue-cultured plants and cutting-propagated plants, respectively. The molecular weight markers in lane 5 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
Figure 8. Densitometer scans of denatured protein profiles of one-month-old tissue of *Betula*, clone 231.  
A. Molecular weight markers (in kd). 
B. Shoot proliferating culture.  
C. Seedlings.  
D. Tissue-cultured plants.  
E. Cutting-propagated plants.
Plate XIII. Denatured protein profiles of one-month-old tissue of *Betula*, clone 247. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, cutting-propagated plants, and shoot proliferating cultures, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
clones had similar protein profiles, which did not closely match either seedling or tissue-cultured plant protein profiles. Proteins represented by bands 3a, 3b, 4a and 4b were present in proportionately greater quantities in seedlings and micropropagated plants than in cutting-propagated plants. Perhaps the most striking difference between the true adult form and juvenile and putative juvenile forms was a 21 to 22 kd protein (labeled 5), which stained heavily in seedlings and tissue-cultured plants, but stained only slightly in cutting-propagated plants. Other differences in banding pattern and/or intensity could be seen in regions of gels labeled 6, 7, and 8, with adult tissue generally displaying more intense staining and a greater number of bands than juvenile tissue.

At four months post-acclimation, all four types of plants (seedling, tissue-cultured, grafted and cutting-propagated) for all three clones showed similar SDS-protein banding patterns (Plates XV-XVII; Figures 11-13). The only consistent difference that could be detected was slightly greater staining of three bands in grafted and cutting-propagated plants. These proteins, collectively labeled region 9, are approximately 66 kd in size.
Plate XIV. Denatured protein profiles of one-month-old tissue of *Betula*, clone 273. Lanes 2 to 5 show profiles of tissue from shoot proliferating cultures, seedlings, tissue-cultured plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
Plate XV. Denatured protein profiles of four-month-old tissue of *Betula*, clone 231. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and \(\alpha\)-lactalbumin (14.2).
FIGURE 11.
Plate XVI. Denatured protein profiles of four-month-old tissue of *Betula*, clone 247. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
Plate XVII. Denatured protein profiles of four-month-old tissue of *Betula*, clone 273. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
PLATE XVII.
FIGURE 13.
Identical banding patterns were observed for all four types of eight-month-old plants of clone 231 (Plate XVIII; Fig. 14). Denatured protein profiles of eight-month-old 231 tissue closely resembled the banding patterns for four-month-old 231 tissue. No distinct differences could be seen for eight-month-old tissue of clone 247 (Plate XIX; Fig. 15), but, for clone 273, grafted plants and cutting-propagated plants had banding patterns that were slightly different than banding patterns of seedlings and tissue-cultured plants (Plate XX; Fig. 16). The differences were primarily in the large molecular weight proteins (>66 kd) and in the region from 55 kd to 25 kd.

The greatest variation in peroxidase activity and isozyme patterns existed at one and eight months post-acclimation. At one month, seedlings and tissue-cultured plants showed little peroxidase activity, while cutting-propagated plants exhibited high activity (Plate XXI). This was consistent across all three clones investigated. Detection of isozymes in juvenile or micropropagated material which differed from those in adult plants was difficult, due to the exceptionally low peroxidase activity in some samples. Faint banding in the region labeled 2, in samples from seedlings and tissue-cultured plants, indicated that the isozymes present may be largely the same as in
Plate XVIII. Denatured protein profiles of eight-month-old tissue of *Betula*, clone 231. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
FIGURE 14.
Plate XIX. Denatured protein profiles of eight-month-old tissue of Betula, clone 247. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and $\alpha$-lactalbumin (14.2).
Figure 15. Densitometer scans of denatured protein profiles of eight-month-old tissue of *Betula*, clone 247. A. Molecular weight markers (in kd).
B. Seedlings. C. Tissue-cultured plants.
D. Grafted plants. E. Cutting-propagated plants.
Plate XX. Denatured protein profiles of eight-month-old tissue of Betula, clone 273. Lanes 2 to 5 show profiles of tissue from seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively. The molecular weight markers in lane 1 are as follows (in kd): bovine albumin (66); ovalbumin (45); glyceraldehyde-3-P dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α-lactalbumin (14.2).
Plate XXI. PAGE-peroxidase patterns of 1-month-old Betula tissue. Lanes 1-3 are from clone 231; lanes 4-6, clone 247; lanes 7-9, clone 273. Within each clone, the three lanes show isozyme profiles of seedling, tissue-cultured, and cutting-propagated plants, respectively.
adult tissue, but less concentrated.

Peroxidase isozyme patterns were similar for all types of plants and all clones at the four-month evaluation time (Plates XXII to XXIV). Clonal differences did exist in the levels of peroxidase activity, with clone 231 exhibiting reduced peroxidase activity. Clone 231 also had the lowest peroxidase activity of the three clones at one month post-acclimation.

The use of summer dormant buds, rather than shoot tips, as a tissue source at the eight-month evaluation time produced numerous new peroxidase isozymes, and differences in banding patterns between plant types. Seedlings, tissue-cultured plants, grafted plants and cutting-propagated plants of all three clones appeared to have four bands in common, in region 2 (Plates XXV to XXVII). Additional fifth and sixth bands were present in this region in clones 231 and 247, but were absent in clone 273. Some of the isozymes in region 2 appeared to be common to all plant types and clones at all evaluation times. Major differences between mature and juvenile forms could be seen in region 1, a group of slower migrating isozymes. Grafted and cutting-propagated plants show identical banding patterns in this region, which are different from the banding patterns of seedlings and tissue-cultured plants. In
Plate XXII. PAGE-peroxidase patterns of 4-month-old *Betula* tissue, clone 231. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXIII. PAGE-peroxidase patterns of 4-month-old *Betula* tissue, clone 247. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXIV. PAGE-peroxidase patterns of 4-month-old Betula tissue, clone 273. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXV. PAGE-peroxidase patterns of 8-month-old Betula tissue, clone 231. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXVI. PAGE-peroxidase patterns of 8-month-old *Betula* tissue, clone 247. Lanes 1–4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXVII. PAGE-peroxidase patterns of 8-month-old Betula tissue, clone 273. Lanes 1–4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
grafted and cutting-propagated plants of clone 231 and 273 showed the same three prominent bands, and two or three weaker bands. Mature-phase plants of clone 247 showed only two isozymes in this region.

Two highly mobile peroxidase isozymes (labeled 3 and 4) were seen in clone 231 at eight months. These two isozymes were nearly absent in seedlings and were found in only low quantities in tissue-cultured plants. Both grafted and cutting-propagated plants had greater quantities of these two isozymes than either seedlings or micropropagated plants.

Differences in esterase isozymes were detected between the various types of plants tested. Two types of esterase isozymes were common: highly mobile isozymes, which were well-resolved; and less mobile isozymes, which appeared as diffuse, broad bands near the tops of the gels. Both general types of esterase isozymes were useful in showing differences between types of plants.

At one month post-acclimation, multiple densely-stained and well-resolved bands could be seen in seedling samples of all three clones (Plate XXVIII). Samples from cutting-propagated plants were characterized by the presence of either a single densely-stained, well-resolved band, or the absence of any well-resolved bands. Isozyme banding patterns of
Plate XXVIII. PAGE-esterase patterns of 1-month-old *Betula* tissue. Lanes 1-3 are from clone 231; lanes 4-6, clone 247; lanes 7-9, clone 273. Within each clone, the three lanes show isozyme profiles of seedling, tissue-cultured, and cutting-propagated plants, respectively.
general, seedlings and tissue-cultured plants exhibited either faint bands of the same isozymes seen in the mature forms, or different faint bands. In region 1, clones 231 and 247 micropropagated plants contained only a single well-resolved band and resembled mature plants with regard to this type of esterase. Tissue-cultured plants of clone 273 exhibited multiple well-resolved isozymes, and were identical to seedlings in this respect. Differences in the slowly migrating, diffuse esterases could also be seen between types of plants.

Consistent differences between types of plants were difficult to detect in tissue collected at four months post-acclimation (Plates XXIX to XXXI). Esterases in seedlings and tissue-cultured plants of clone 273 were similar to each other, and contrasted the lack of esterases in grafted and cutting-propagated plants.

The use of summer dormant buds at the eight-month evaluation time resulted in the detection of additional esterase isozymes, but differences in banding pattern between plant types were not common (Plates XXXII to XXXIV). The broad esterase bands were stained more intensely in samples from seedlings and tissue-cultured plants of clones 231 and 273 than in grafted or cutting-propagated plants of the same clones. In clone 247, the pattern of well-resolved isozymes seen in
Plate XXIX. PAGE-esterase patterns of 4-month-old Betula tissue, clone 231. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXX. PAGE-esterase patterns of 4-month-old Betula tissue, clone 247. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXXI. PAGE-esterase patterns of 4-month-old *Betula* tissue, clone 273. Lanes 1–4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXXII. PAGE-esterase patterns of 8-month-old *Betula* tissue, clone 231. Lanes 1–4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXXIII. PAGE-esterase patterns of 8-month-old Betula tissue, clone 247. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
Plate XXXIV. PAGE-esterase patterns of 8-month-old Betula tissue, clone 273. Lanes 1-4 show profiles from tissue of seedlings, tissue-cultured plants, grafted plants, and cutting-propagated plants, respectively.
seedlings was different than the pattern seen in tissue-cultured, grafted or cutting-propagated plants.

Discussion.

Protein banding patterns resulting from SDS-PAGE of tissue from one-month-old seedlings and tissue-cultured plants were identical to each other, but different from protein banding patterns of adult tissue collected at the same time. These data indicate that, at least initially, birch plants derived from shoot proliferating tissue cultures initiated from shoot tips of flowering trees may be more like seedlings than mature trees. The existence of protein profiles in micropropagated plants, which are unlike those in adult forms, suggest that tissue-cultured plants have a physiology which is different from adult trees. Changes in physiology, coupled with the acquisition of juvenile morphology, suggest that in vitro culture may induce rejuvenation in mature clones of Betula. SDS-PAGE has been shown to be a sensitive method for detection of physiological changes which are associated with changing morphology (Ellis and Judd, 1987).

The lack of any striking differences in four- or eight-month-old tissue of any of the variously propagated plants indicates that after four months of
growth, seedlings and tissue-cultured plants are attaining a physiology similar to adult plants.

Several proteins which were seen in samples from one-month-old seedling and tissue-cultured plants, but not in samples from adult plants, may be intimately involved in phase change. Further investigation of these proteins may result in protein markers which could be useful in measuring relative levels of juvenility or maturity in plant tissue. Fukasawa (1966) found that electrophoretic separation of basic proteins of Hedera helix tissue cultures in the adult and juvenile form revealed some differences in protein banding. Some bands were denser in mature callus and one band was denser in juvenile callus.

At one month post-acclimation, true juvenile forms and putative tissue culture revertants could be distinguished from adult forms by the level of peroxidase activity, even though striking differences in isozyme banding were not apparent. Trippi (1963c) found that peroxidase activity tended to increase with age in Robinia pseudoacacia. Changes in peroxidase activity have also been reported to be useful as biochemical markers of the reacquisition of seedling morphology and rooting capacity in globe artichoke (Moncousin, 1982). Peroxidase has been shown to be important in tree lignification, through its
involvement in polymerization of $p$-coumaryl alcohols to lignin (Harkin and Obst, 1973). It is likely that lignification is occurring to a lesser degree in young seedlings and tissue-cultured plants, than in adult trees. High levels of peroxidase may, therefore, be unnecessary in young and relatively succulent plants.

Evaluation of shoot tip tissue of seedlings, tissue-cultured plants, grafted plants and cutting-propagated plants at four months revealed no differences in peroxidase isozymes or activity within a clone. These results correlate with those seen in SDS gels, indicating that juvenile forms mature significantly by four months post-acclimation. The reappearance of detectable differences in peroxidase isozyme banding at eight months is likely due to the use of summer dormant buds rather than shoot tips as a tissue source. It has been shown that differences in isozymes may be seen in one type of tissue and not in others on the same plant (Allichio, et al., 1987). Summer dormant buds of *Betula* appear to contain more isozymes of both peroxidase and esterase than growing shoot tips. Although a greater number of peroxidase isozymes were seen in adult birch tissue than in juvenile tissue, Berthon and coworkers (1987) found that juvenile, rooting cuttings of *Sequoiadendron*
exhibited more acidic peroxidases than mature microcuttings.

Morphological evaluation of mature, juvenile and tissue-cultured plants (Chapter II) provided evidence supporting the idea that adventitiously-produced shoots were more likely to be rejuvenated than shoots arising from release of axillary buds. Tissue-cultured plants of clone 231 (which multiplies slowly via axillary release) initially acquire fewer juvenile features, and redevelop some mature characteristics more rapidly than micropropagated plants of the other two clones, which multiply largely through adventitious shoots). No correlation can be seen, however, between the mode of shoot proliferation and the level of rejuvenation indicated by denatured or native protein banding patterns. Analysis of peroxidase and esterase isozymes, as well as denatured proteins, indicates that seedlings and micropropagated plants are nearly identical to mature forms by four months post-acclimation. In contrast, morphology of true juvenile and tissue-cultured plants at four months suggests that full maturity has not yet been attained (Chapter II). The apparent lack of correlation between the expression of juvenility in morphology and in protein profiles at some evaluation times may result from the use of tissue which is not capable of exhibiting protein differences
at those particular times. The increase in isozyme differences of some samples between four and eight months, for example, may be due to the use of summer dormant buds at the eight month evaluation, rather than the shoot tips used at the one and four month times, and not to the existence of increased juvenility in those samples.

Analysis of proteins in four- and eight-month-old grafted plants showed them to have banding patterns identical to true mature types. Protein analysis performed within the first month of scion growth may yield some protein differences indicating partial rejuvenation of the mature scion. The results of this study indicate that in vitro culture of birch is more likely to cause rejuvenation than grafting mature scions on juvenile rootstocks. The nature and extent of in vitro rejuvenation is unknown and significant efforts will be necessary to illucidate the biochemical events controlling the complex process of phase change in woody perennials.
List of References.


Trippi, V. S. 1963c. Studies on ontogeny and senility in plants. IV. Activity of some enzymes at different stages of ontogeny and in clones from juvenile and adult zones of *Robinia pseudoacacia* 20(2):160-166.

Phase change is a major physiological event which occurs in woody perennials and controls the ability of tissue to flower, the adventitious rooting ability of vegetative propagules and the morphology of given plant parts. Seedling and adult birch exhibit distinct morphology for the following features: bark color, presence of flowers, pubescence, anthocyanin development, stem diameter, and leaf size and shape. Tissue-cultured plants (originally mature) initially have morphology which is more similar to seedlings, and generally unlike that of mature trees. Tissue-cultured plants probably are not as juvenile as seedlings, since acquisition of mature features occurs more rapidly in micropropagated plants than in seedlings. Grafting mature scions onto juvenile rootstocks appears to cause a transient reversal to juvenile morphology. Rejuvenation by this procedure is not as stable as the rejuvenation which occurs via tissue culture. The small size of the mature scion may be necessary to cause any substantial rejuvenation. Experiments
comparing rejuvenation when large and small scions are used may answer this question.

Correlation between high levels of adventitious shoot formation and rejuvenation appear to exist, but the effect varies some depending on the genotype. It would be beneficial to compare the level of juvenility in shoots which arose from axillary breaks to adventitious shoots from the same shoot clump.

Evaluation of total denatured protein banding patterns show that initially tissue-cultured plants are more like seedlings than adult plants. Direct correlations between detectable total denatured protein differences do not always exist. It is unclear why strong correlations can not be seen, but it is understandable that changes in morphology lag behind biochemical changes. Some interesting possibilities exist for further research to better characterize those proteins seen in juvenile tissue, but not in adult tissue. Two-dimensional PAGE of these proteins could provide information useful in determining how these proteins are involved in phase change.

Peroxidase and esterase isozyme analyses are also useful in detecting differences in isozyme activity and composition between the variously-propagated plants. The type of tissue that is evaluated is critical in determining if isozyme banding differences can be
seen. Thorough studies should be conducted to determine what tissues are best for use in detecting phase related protein differences. Further investigations of peroxidase and esterase isozymes may yield more information if isoelectric-focusing PAGE is employed. Examination of RNA profiles would be useful in evaluating phase change associated changes at the transcription level. The biochemistry of phase change is still unknown and significant efforts, conducted under closely controlled environmental conditions, will be necessary to make progress in this area.
LIST OF REFERENCES


Trippi, V. S. 1963a. Studies on ontogeny and senility in plants. II. Seasonal variation in proliferative capacity in vitro of tissues from branches from juvenile and adult zones of *Aesculus hippocastanum* and *Castanea vulgaris*. Phyton 20(2):146-152.

Trippi, V. S. 1963b. Studies on ontogeny and senility in plants. III. Changes in the proliferative capacity in vitro during ontogeny in *Robinia pseudoacacia* and *Castanea vulgaris* and in adult and juvenile clones of *R. pseudoacacia*. Phyton 20(2):153-159.

Trippi, V. S. 1963c. Studies on ontogeny and senility in plants. IV. Activity of some enzymes at different stages of ontogeny and in clones from juvenile and adult zones of *Robinia pseudoacacia* 20(2):160-166.


