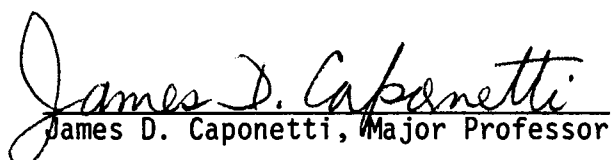
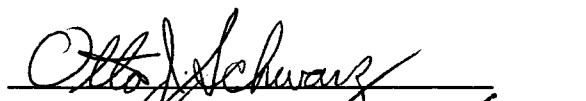


To the Graduate Council:

I am submitting herewith a dissertation written by Thomas E. Byrne entitled "Morphogenesis in Several Cultivars of Boston Fern." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.


James D. Caponetti, Major Professor

We have read this dissertation
and recommend its acceptance:






Accepted for the Council:


The Graduate School

MORPHOGENESIS IN SEVERAL CULTIVARS OF
BOSTON FERN

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Thomas E. Byrne

June 1984

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ABSTRACT

Stolon tips from sporophytes of Nephrolepis exaltata c.v. 'bostoniensis' and its cultivars N. exaltata c.v. 'scotti' and N. exaltata c.v. 'dwarf boston' were aseptically cultured on Murashige Fern Multiplication Medium modified by the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) 0.5-4.0 mg/l. Callusing occurred in the apical and lateral buds of the stolon tips in four to six weeks. Histological sections demonstrated that the callus originated from the provascular tissue of the apical meristems of the stolon buds. Basal medium with 3% sucrose and 0.5 mg/l 2,4-D produced the greatest increase in fresh weight of subcultured callus tissue in all cultivars. Organogenesis into shoots and roots from undifferentiated callus tissue occurred on basal medium with combinations of 5×10^{-7} M kinetin (K) plus 5×10^{-7} M naphthalene acetic acid (NAA), 10^{-6} M K plus 10^{-5} M NAA and 10^{-6} M K plus 5×10^{-7} M NAA in 12 weeks. Attempts to induce organogenesis with indole-3-acetic acid (IAA) and isopentenyl adenine (2ip) produced only roots at all hormone levels tested. Viable suspension cultures were produced from stolon callus of all cultivars employing a liquid basal medium containing 0.5 mg/l 2,4-D. Callus clones from single cells in suspension cultures were obtained by the Bergmann Technique. The clones were transferred to morphogenic medium and formed complete new plants. Thus, the morphogenic totipotency of pteridophytes has been achieved with the production of whole sporophytes

from callus tissue and single cells of three Nephrolepis cultivars.

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I. INTRODUCTION

In plants capable of sexual reproduction, a diploid zygote formed at fertilization will undergo cell division to produce an embryo and ultimately form tissues and organs. The daughter cells of the fertilized ovum will in subsequent divisions express their phenotypic potential to a lesser degree as cells undergo differentiation to specific tissue types. Nevertheless single cells of a meristematic region of a shoot or root tip, with appropriate tissue culture techniques, are capable of acting like a zygote and developing into a complete new plant. This concept of cellular totipotency was proposed by Haberlandt (1902), and has been realized with improved in vitro plant tissue culture techniques.

The morphogenetic totipotency of some angiosperm sporophytes is well known. Under appropriate conditions, carrot root phloem cells have the capacity to produce pseudo-embryos which can differentiate into normal sporophytes. Numerous experiments along this line have been conducted by F. C. Steward and his associates on horticultural cultivars of carrots during the 1950's (Steward et al., 1958a; Steward et al., 1958b; and Steward and Shantz, 1959). Tobacco stem pith has the capacity to produce callus which can differentiate into normal sporophytes. F. Skoog and T. Murashige (1962) and their associates have conducted numerous experiments on several varieties and hybrids of tobacco since the mid 1950's (Murashige and Skoog, 1962). These are but two examples. Other

angiosperms are under investigation. The hypocotyl of pine seedlings has the ability to produce callus which can differentiate into sporophytes. Sporophytic plantlets have also been obtained from embryos and needles. C. L. Brown and several others have conducted numerous experiments on several species of pine since the early 1970's (Reilly and Brown, 1976). If the principles of totipotency are valid, they should apply to other plant groups besides angiosperms and gymnosperms. The morphogenetic totipotency of pteridophytes has not yet been achieved. Limited callus induction and some tissue differentiation in ferns and in some other pteridophytes has been accomplished to some extent. Sporophytic leaf blade tissue has been produced on basal medium with sucrose from callus of Pteris cretica (Bristow, 1962), Pteris vittata (Kato, 1963) and Cyclosorus dentatus (Mehra and Palta, 1972). However, gametophytic tissue was produced from callus of the same organisms when the basal medium contained no sucrose. The production of whole sporophytes from callus has not yet been realized.

A review of the literature concerning callus formation in pteridophytes begins with Beyerle's (1932) description of spontaneous callus formation in Pteris tremula from the leaves of sporeling plants maintained on damp soil. The first report of in vitro pteridophyte callus induction was from Morel and Wetmore (1951) who observed the spontaneous formation of callus from gametophytes of Osmunda cinnamomea on Knudson's Medium. Steeves and Sussex (1952, 1955) and Sussex and Steeves (1953) observed callus induction

from gametophytes of Pteridium aquilinum var. latiusculum and recorded the production of different callus forms with alteration of the medium from Knudson's Solution with Berthelot's Trace Elements to Knop's Medium with 1% dextrose. Morel (1956a, 1956b) using Knop's Medium supplemented with glucose reported the callus of Adiantum pedatum was capable of differentiation into sporophytic leaf tissue, and reported that callus tissues from stem sections of Selaginella and Osmunda cinnamomea could also be cultured on Knop's Medium. The fern Marsilea vestita (Laetsch and Briggs, 1961) was induced to produce callus from shoot apices on Knop's Medium supplemented with kinetin. Bristow (1962) employing White's Medium plus 2,4-D produced callus from leaf blade tissue of Pteris cretica and produced gametophytic and sporophytic tissues with different sucrose concentrations. Kato (1963) observed the production of callus in Pteris vittata during gametophytic development on Moore's Medium with Nitsch's Trace Elements plus 2,4-D. DeMaggio (1964) employing Moore's Medium with added coconut milk reported that callus from Lycopodium obscurum gametophytes could be maintained in an undifferentiated state and that sporophytic bud tissues could be obtained from gametophytic callus but did not form whole plants. Peterson (1967) initiated the production of callus in Ophioglossum petiolatum bud primordia by mechanically wounding the primordia and by placing excised primordia on Murashige and Skoog Medium plus kinetin and 2,4-D. Mehra and Palta (1972) reported callus production from roots of Cyclosorus dentatus cultured on Knudson's

Medium with 2,4-D and also produced gametophytic and sporophytic leaf tissues with different sucrose concentrations. The fungicide, benlate (Schedlbauer, 1978) induced callus on gametophytes of Ceratopteris thalictroides grown on Thompson's Medium. Kshiragar and Mehta (1978) observed callus initiation from rhizome explants of Pteris vittata when cultured on White's Medium supplemented with sucrose, coconut milk and 2,4-D. Mahabale and Patankar (1980) recorded the growth of callus from Drynaria querifolia gametophytes grown on Knudson's Medium with Nitsch's Trace Elements. Partanen, Sussex, and Steeves (1955) developed a series of mutant callus cultures from Pteridium aquilinum gametophytes termed "tumourous" by the exposure of spores to 25 kR of X-rays.

The Boston series of ferns (Nephrolepis exaltata c.v. 'bostoniensis') have been popular ornamental plants for many years. During the 1970's two million Boston ferns were sold each year in the United States (Perl, 1977). These ferns are sterile except for Nephrolepis exaltata c.v. 'bostoniensis' "var. fertilis." Non-fertile cultivars of Boston ferns are propagated in the greenhouse by simple layering of runners (stolons) which originate from the base of the petioles, take root and form new plants (Morton, 1958). Recent advances in sterile tissue culture methods have made possible the production of large numbers of plantlets by inducing stolon tip multiplication in Boston fern (Harper, 1976) and Fishtail fern (N. falcata c.v. 'furcans') (Beck and Caponetti, 1983). Many nurseries are now producing large numbers of Boston

fern in tissue culture from excised stolon tips rather than by greenhouse propagation of layered stolon tips.

The capacity for rapid propagation and general amenability of Boston and Fishtail ferns in sterile tissue culture has led to the idea that Nephrolepis could serve as a model system for beginning studies of morphogenesis in pteridophytes. A study of the induction, proliferation, and differentiation of callus and free cells of Nephrolepis exaltata and some of its cultivars would serve to answer some basic questions in the morphogenesis of ferns. Complete differentiation of whole plants from undifferentiated cells and tissues has not been accomplished yet with pteridophytes. In addition, these basic studies could eventually supply ornamental horticulturists and the nursery trade with new and improved methods for the rapid propagation of the Boston fern group which are popular as ornamental plants.

The purpose of this investigation is to elucidate the facts concerning induction, proliferation and differentiation of callus and free cells into whole plants from leaf and stem tissues of several Boston fern cultivars. Certain aspects of leaf and stem anatomy were examined in section in order to study the anatomical similarities and differences between cultivars, and to determine the point of origin of callus and free cells in each cultivar. Such information would be useful in determining the morphogenesis of callus and free cells.

II. MATERIALS AND METHODS

Stock Plant Sources

Dwarf cultivars were obtained from Dr. Bruce McAlpin at the New York Botanical Garden, Bronx, New York. The cultivars are Nephrolepis exaltata c.v. 'scotti' 557-76 and N. exaltata c.v. 'dwarf boston' 3241-78. Upon returning to Knoxville, all plants were repotted and placed in the University of Tennessee, Knoxville, Botany Department greenhouse. Stock cultivars of Boston fern, Nephrolepis exaltata c.v. 'bostoniensis' were available from a collection in the Botany Department greenhouse obtained several years ago from North Carolina Farms, Indian Trail, North Carolina.

Microtechnique of Leaf and Stem Tissues

Leaf blade and petiole, rhizome, and stolon tip segments were killed and fixed in three fixative solutions based on the advice of Dr. E. Graham. The first fixative solution was Craff which was composed of 3 ml of 1% chromium oxide, 2 ml of 10% acetic acid, 1 ml of 20% formaldehyde and 4 ml of distilled water. The second fixation solution was Glutaraldehyde Fixative which consists of 5% glutaraldehyde in distilled water. The third fixation solution was FAA Fixative composed of 90 ml of 50% ethyl alcohol, 5 ml of glacial acetic acid and 5 ml of 40% formalin (Jensen, 1962). All three fixation solutions were allowed 24 hours contact time with the tissues.

Dehydration from the fixed and hardened tissues were conducted as follows: for Craff and glutaraldehyde fixation solutions, an alcohol change was made every 30 minutes, with ethyl alcohol concentrations of 5%, 10%, 20%, 30%, and 40%. After completion of this series, 30 minute intervals changes of tert-butyl alcohol were made in the following concentrations: 50%, 60%, 70%, 80%, 90%, 95%, 100%, 100%, and 100%. FAA fixative solution was dehydrated in 30 minute intervals beginning with 50% tert-butyl alcohol and proceeding to 100% tert-butyl alcohol with three changes at 100% (Jensen, 1962).

The fixed and dehydrated tissue was infiltrated and embedded with Fisher Tissue Prep. All sections were cut on an American Optical Rotary Microtome at a thickness of 10 microns. Hematoxylin dye with an iron alum mordant was employed to stain all tissue sections (Jensen, 1962).

Callus Induction from Tissues

Leaf blade tissue was sectioned into 1 cm squares, petiole tissue was sectioned into 2 cm lengths, rhizome tissue was sectioned into 2 cm lengths, and stolon tips of 2 cm lengths were each disinfected by the following procedure. The sectioned tissues were placed in a 150 mm x 25 mm screw cap test tube containing 25 ml of a 1% Alconox solution for 2 minutes. After the Alconox solution was decanted, 30 ml of a 10% Clorox solution was added to the test tube. The test tube was gently agitated for 10 minutes. In a

UV sterilized room, the tissue was transferred to a sterile Petri dish containing sterile distilled water. The 1 cm square leaf blade tissue was placed on callusing medium adaxial side up without being further dissected. The 2 cm sections of petiole, rhizome and stolon tip tissues were then cut to 1 cm lengths and transferred to sterile Murashige Fern Multiplication Medium as modified for fern callusing (Table I). The original medium was previously reported by a former graduate student of Murashige (Harper, 1976). Appropriate stock solutions of the compounds were mixed together for each liter of medium prepared. The media were gelled with 10.0 gm per liter of Bacto-Agar (Difco Laboratories). After the pH was adjusted to 5.7 using KOH and HCl solutions, media were placed in 25 x 150 mm Pyrex glass culture tubes at the rate of 20 ml per tube, plugged with cotton, each capped with a plastic Kaput, and steam sterilized at 15 psi for 20 minutes.

Cultures containing tissue segments were incubated in a walk-in culture room where the temperature was set at 25 degrees Centigrade. White light was furnished by a combination of fluorescent tubes and incandescent bulbs at an intensity of 2500 lux at the level of the cultures in a 16 hour light, 8 hour dark photoperiod. Cultures for callus initiation in the dark were placed in a light tight wooden box and placed in the same culture room as those cultures in the light.

Leaf blade, petiole and rhizome segments did not produce callus under these cultural conditions but stolon tips did.

Table I. Murashige Fern Multiplication Medium as Modified
for Fern Callusing.

Compound	Mg/1 Medium
NH_4NO_3	1,650.00
KNO_3	1,900.00
CaCl_2	332.00
MgSO_4	181.00
KH_2PO_4	170.00
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	255.00
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	37.00
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.80
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.90
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.60
H_3BO_3	6.20
KI	0.83
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
CuSO_4	0.016
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
Thiamine HCl	0.40
Myo-inositol	100.00
2,4-D	2.00
Sucrose	30,000.00
Bacto-Agar	10,000.00
pH adjusted to 5.7	

Therefore, stolon tips were placed into a series of media containing several combinations of sucrose and hormones in order to determine which combination produced the greatest fresh weight of callus. Based on information from Hildebrandt and Riker (1953), sucrose at concentrations of 1, 2, 3, and 4% were combined with 2,4-D concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg per liter based on the 2,4-D experiments of Mehra and Palta (1972). Each concentration of 2,4-D was tested with each concentration of sucrose making a total of 32 combinations that were tested on all three cultivars. Means, standard deviation, analysis of variance and Duncan's Multiple Range Test were calculated for each series of replications in accordance with the method of Barr (1976).

Callus Differentiation into Whole Plants from Stolon Callus

In order to induce the differentiation of callus tissue into whole plants, Murashige Fern Multiplication Medium was modified by the addition of varying concentrations of naphthalene acetic acid (NAA) and kinetin (K) as suggested by Beck and Caponetti (1983). In a first series of experiments, hormone concentrations were K 0, $5 \times 10^{-7}M$, $1 \times 10^{-6}M$, $5 \times 10^{-6}M$, $1 \times 10^{-5}M$, and $5 \times 10^{-5}M$, and NAA 0, $5 \times 10^{-7}M$, $1 \times 10^{-6}M$, $5 \times 10^{-6}M$, $1 \times 10^{-5}M$, and $5 \times 10^{-5}M$. Each concentration of K was tested with each concentration of NAA for a total of 36 combinations with each of the three cultivars. In a second series of experiments, isopentenyl adenine (2ip) in concentrations of 0, $5 \times 10^{-7}M$, $1 \times 10^{-6}M$, $5 \times 10^{-6}M$, $1 \times 10^{-5}M$, and $5 \times 10^{-5}M$ were tested

with indole-3-acetic acid (IAA) concentrations of 0, $5 \times 10^{-7}M$, $1 \times 10^{-6}M$, $5 \times 10^{-6}M$, $1 \times 10^{-5}M$, and $5 \times 10^{-5}M$ as suggested by Dr. Schwarz. Each concentration of 2ip was tested with each concentration of IAA for a total of 36 combinations with each cultivar. Each liter quantity of medium was prepared as for callus induction experiments, pre-sterilized in 1500 ml cotton plugged Erlenmeyer flasks, and poured while hot in 100 x 15 ml, pre-sterilized, plastic Petri dishes, 30 ml per dish. After the media gelled completely, callus pieces about the size of a small garden pea seed were transferred by sterile technique from stolon callus cultures, one piece per dish. Data was collected when plantlet differentiation occurred in 12 to 16 weeks. Means, standard deviations, analysis of variance and Duncan's Multiple Range Test were calculated for the data according to the method of Barr (1976).

Callus Induction from Cell Suspension Cultures

Murashige Fern Multiplication Medium as modified by the addition of 0.5 mg per liter of 2,4-D was used in liquid form (without agar). A volume of 50 ml of medium was placed in a series of 250 ml cotton plugged Erlenmeyer flasks and steam sterilized at 15 psi for 20 minutes. Under sterile conditions suspension cultures were initiated by the transfer of 4-5 grams of callus from the culture tubes into each flask. Agitation of the inoculated flask occurred on a horizontal platform shaker (Lab Line Orbit) at 100 revolutions per minute. Suspensions of cells

were subcultured following the technique of Davey (1971) by the transfer of 10 ml of suspension to 50 ml of new medium in 250 ml flasks every 3-4 weeks. An inoculation of 20,000 cells per mm^3 was made for each cultivar after dilution by fresh medium (Thomas and Davey, 1975). Two measurements of growth were made for each suspension culture. The first measurement was packed cell volume (Henshaw, 1966) in which a known volume of suspension was transferred to a 15 ml graduated conical centrifuge tube and centrifuged at $1625 \times g$ for 5 minutes (IEC Clinical Centrifuge). The second measurement was a count of cell numbers determined by using an American Optical Bright Line hemacytometer according to the method of Benson and Gunstream (1976).

Suspensions of single cells and small cellular aggregates were prepared by the Bergmann Technique (1960) in order to establish callus clones. In this plating technique, filtered suspensions of exponentially growing cells, containing approximately 2,000, 1,000, 500, 250, and 125 cells per Petri plate were mixed with a thin film of Murashige Fern Multiplication Medium as modified by the addition of 0.5 mg/l, 2,4-D. The lower layer of agar contained a liquid basal medium with 0.5 mg/l 2,4-D, that had been filtered from an exponentially growing suspension culture. This cell free filtrate was warmed (45 degrees Centigrade) and aseptically added to melted agar (45 degrees Centigrade) to form the "conditioned" (Stuart and Street, 1969) lower layer of the Bergmann plate. Cell clusters were marked with red and blue

colored felt tip pens on the surface of the Petri dish in order to observe the progress of single cell growth and not confuse cellular aggregates with single cells. All Petri plates were placed in the culture room for callus induction to occur. After callus clones were obtained, callus differentiation into whole plants was accomplished. Clones were placed on basal medium with K and NAA in order to induce organogenesis.

III. RESULTS

Anatomy of Leaf and Stem Tissues

A histological examination of leaf and stem tissues of the three cultivars was performed to note similarities and differences between cultivars as an aid in further studies of callus origin and proliferation. Tissues were killed and fixed in three different fixation solutions, namely Craff Fixative, 5% Glutaraldehyde Fixative, and FAA Fixative. Preliminary experiments demonstrated that Craff Fixative provided the best fixation with the least damage to the tissue. Glutaraldehyde produced fractures and gaps between cell walls which were attributable to improper hardening action (Dr. Graham, personal communication), causing the tissue to crack when being sectioned. FAA Fixative produced cellular shrinkage due to dehydration with alcohol.

The anatomy of leaf and stem tissues was essentially the same in all three cultivars. Photomicrographs which display the morphology of the tissues are presented as typical examples for the cultivars under study. A longitudinal section of a stolon apical meristem displays several tissue layers. Plate I, Figures 1 and 2, demonstrate the generally observed position of the apical initials, apical zone, subapical zone, parenchymal tissue, pro-vascular tissue and scales. Espagnac (1973) observed similar correlations in morphological studies of Nephrolepis biserrata (Sw.) Schott. These tissue layers were consistent in the stolons

Plate I. The Apical Meristem.

Figure 1. Longitudinal section of a stolon apical meristem of Nephrolepis exaltata c.v. 'dwarf boston' (200 x).

Figure 2. Schematic representation of the stolon apical meristem, AI, apical initials; AZ, apical zone; SZ, subapical zone; P, parenchyma tissue; PV, provascular tissue; S, scales.

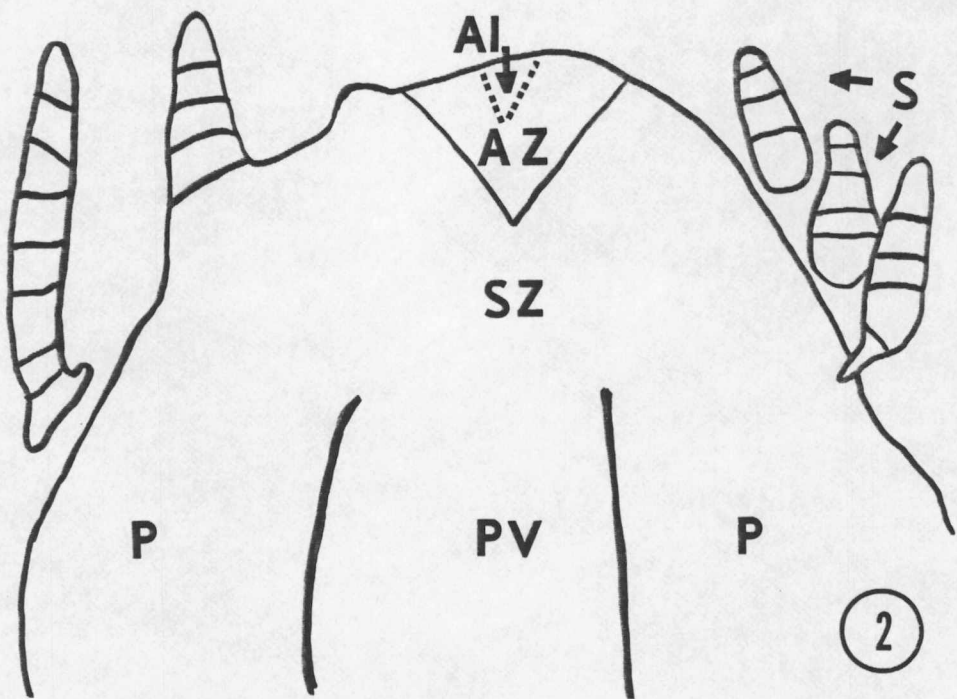
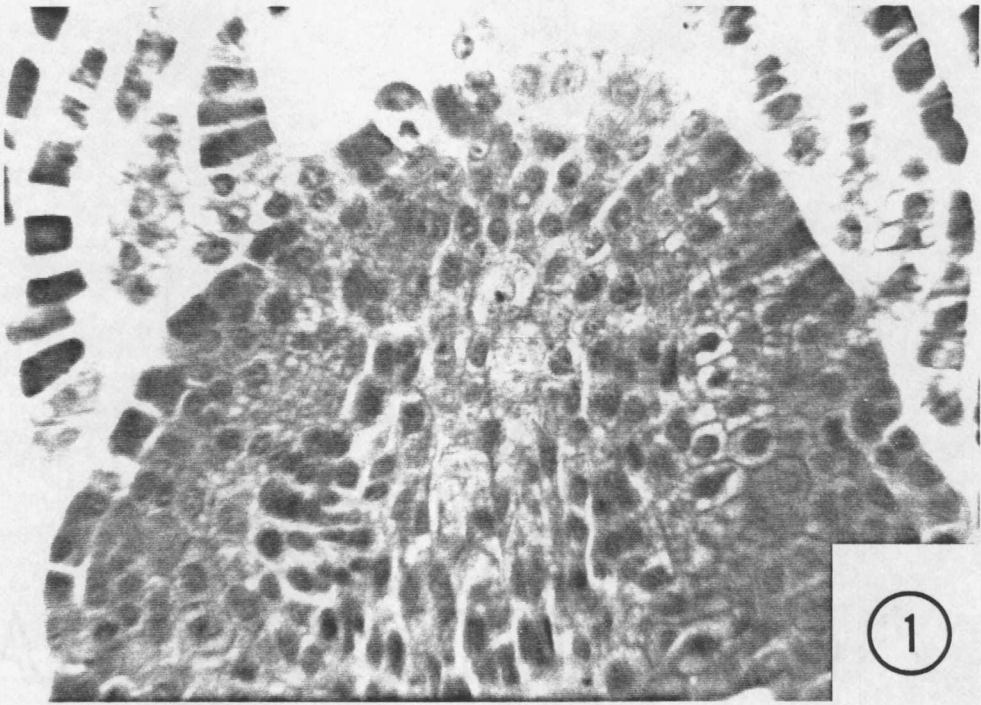


Plate I

of all cultivars. The presence of lateral bud primordia was common in stolons of all cultivars (Plate II, Figures 3 and 4).

The anatomy of leaf tissue was simple in structure, with an upper and lower epidermis enclosing uniform mesophyll tissue with centrally located vascular tissue of the main and lateral veins. This was observed in all cultivars (Plate III, Figures 5 and 6). Each vein in cross section consists of primary xylem overlaying primary phloem enclosed by several layers of sclerenchyma and surrounded by a vein sheath of parenchyma. The anatomy of the rhizome was also similar in all cultivars. The vascular tissue was in the form of a dictyostele with leaf traces consisting usually of three strands as shown in Plate IV, Figures 7 and 8. A detailed view of a vascular bundle (Plate IV, Figure 8) displays the anatomy observed throughout the study. The endodermis and pericycle are clearly observable despite separation due to the effects of fixation. In the central area of the vascular bundle the primary xylem is clearly evident and is surrounded by primary phloem. A ground tissue of parenchyma surrounds each of the vascular strands. This description corresponds to that of Haupt (1953) on general fern rhizome anatomy.

Callus Induction from Tissues

Several tissues of the three Nephrolepis cultivars were tested for their capacity to produce callus. Since callus was obtained from leaf blade tissue in Pteris cretica (Bristow, 1962)

Plate II. Lateral Bud Primordia.

Figure 3. Longitudinal section of a stolon tip of Nephrolepis exaltata c.v. 'scotti' demonstrating the position of two lateral bud primordia (80 x).

Figure 4. Detailed view from Figure 3 of a lateral bud primordium (200 x).

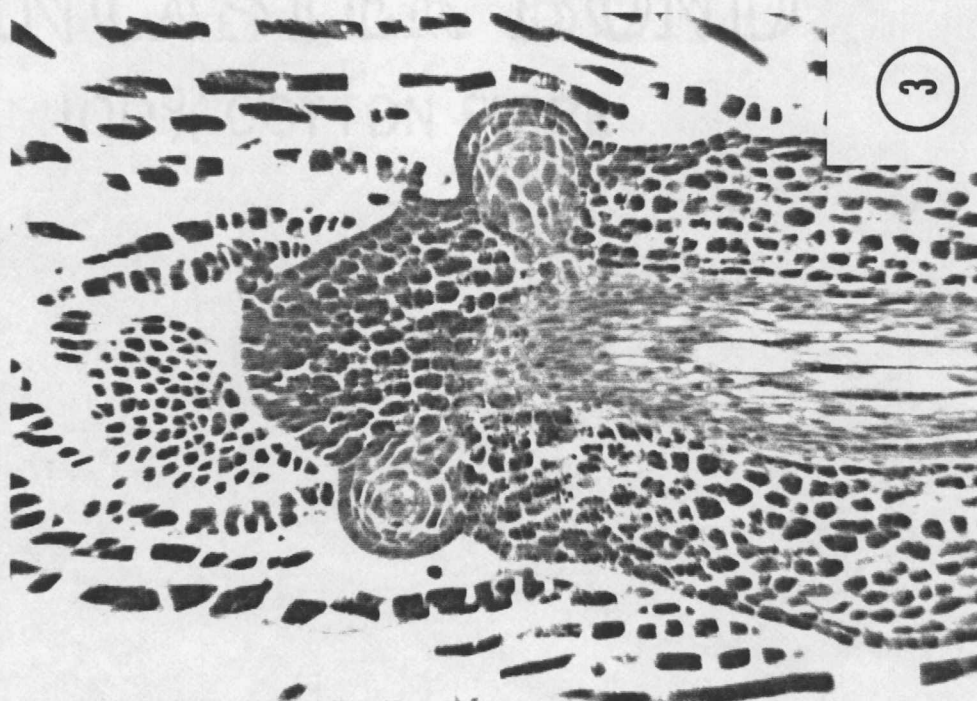
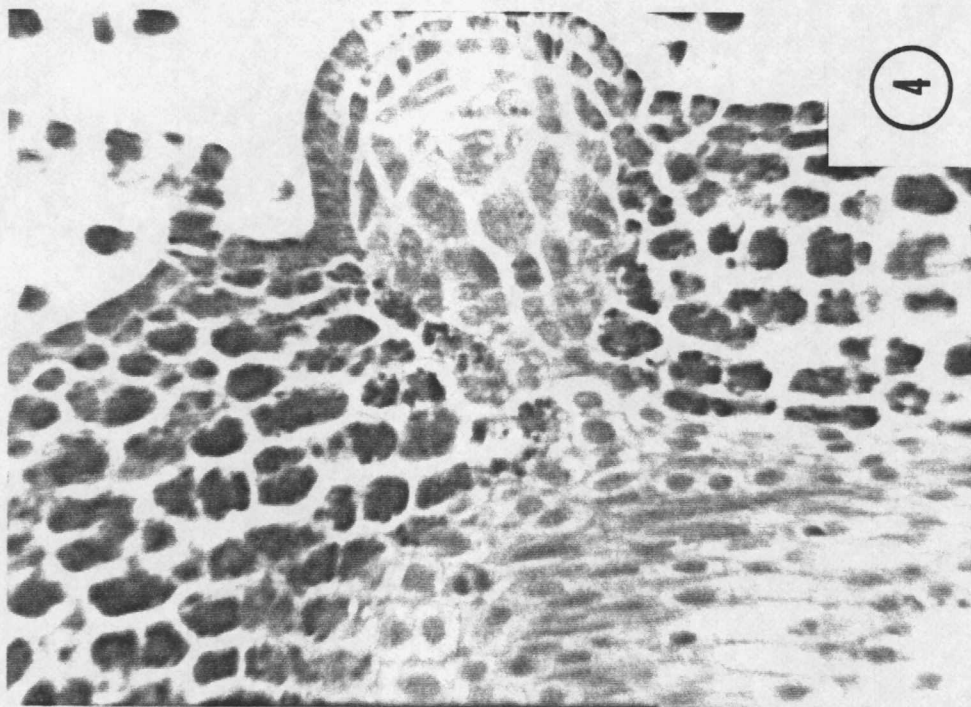
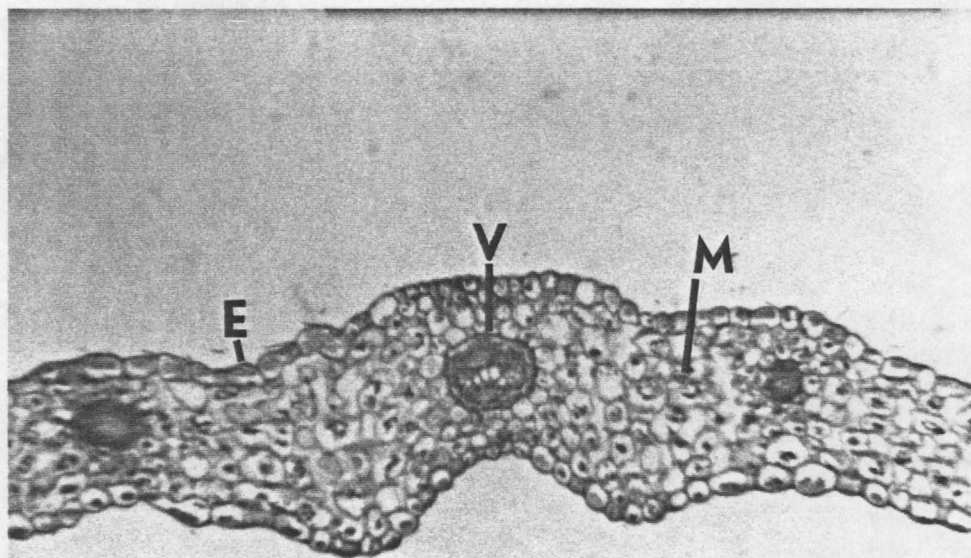


Plate II

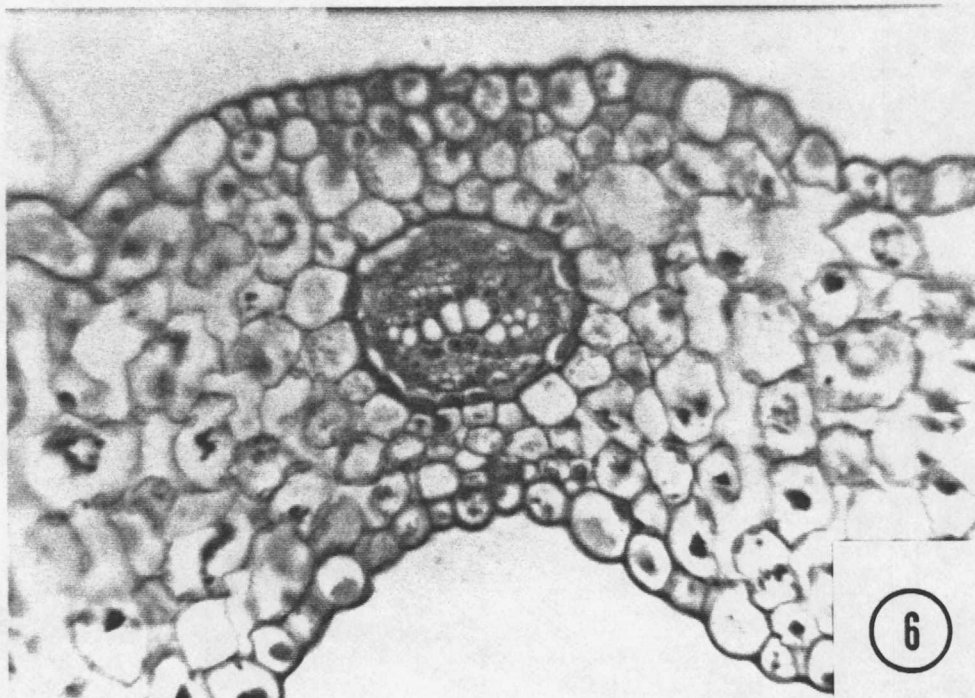
Plate III. The Leaf.

Figure 5. Cross section of a leaf of Nephrolepis exaltata c.v. 'bostoniensis' showing the upper and lower epidermis, E; mesophyll tissue, M; and vascular tissue, V (80 x).

Figure 6. Detailed view from Figure 5 showing the upper and lower epidermis, mesophyll, and vascular tissue in the main vein (200 x).



5



6

Plate IV. The Rhizome.

Figure 7. Cross section of a rhizome of Nephrolepis exaltata c.v. 'dwarf boston' showing three strands of vascular tissue (80 x).

Figure 8. Cross section of a rhizome of Nephrolepis exaltata c.v. 'bostoniensis' demonstrating the position of xylem, X; phloem, Ph; pericycle, Pe; endodermis, E; and parenchymal tissue, P (200 x).

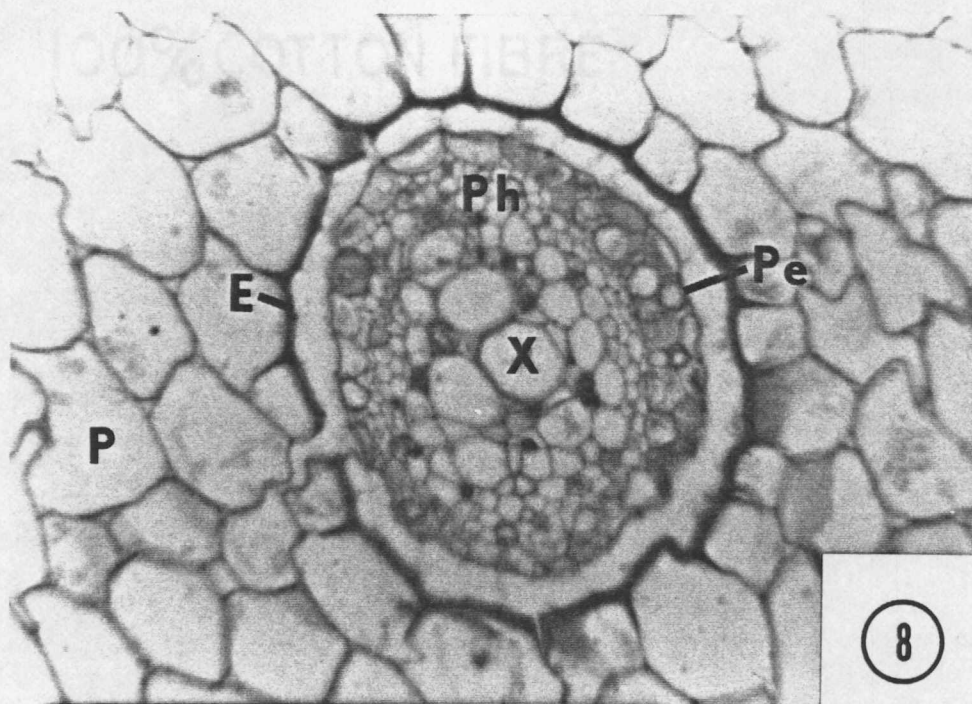
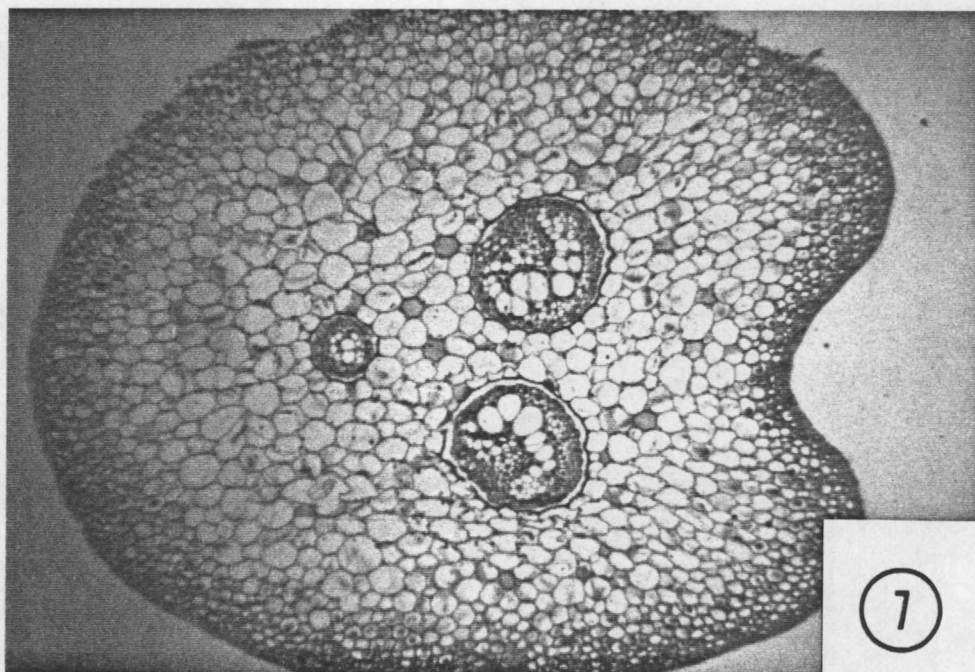


Plate IV

and Pteris vittata (Kato, 1963), an attempt was made to do the same with Nephrolepis. Leaf blade tissue, leaf petiole and rhizome slices 2 cm long failed to initiate callus in cultures maintained in the light and the dark. Attempts to induce callus from stolons was a new concept and callus was induced from apical and lateral bud meristems on media containing various sucrose and 2,4-D concentrations only in cultures maintained in the light.

The callus from stolons of N. exaltata c.v. 'bostoniensis' and its cultivars was chlorophyllous with the pigmentation being uniform. On media with the lower concentrations of 2,4-D (0.5 and 1.0 mg/l) the callus was of a soft nodular texture and faster growing while with higher concentrations of 2,4-D (3.5 and 4.0 mg/l) the callus was of a smooth and extremely friable texture and slower growing. At other concentrations of 2,4-D, the callus displayed morphologies between these two extremes. The callus at all concentrations of 2,4-D tested had a soft to extremely friable texture that allowed for easy subculturing and induction of suspension cultures later.

The histological origin of callus in stolon tips proved to be provascular tissue in both terminal and lateral buds (Plate V, Figures 9 and 10). In examination of Plate V, Figures 9 and 10, one will note that a marked reduction in cell size is observed in the callus induced cells (arrow) due to a rapid mitotic rate typical of meristematic tissue. Mitotic figures were observed in large numbers in the sections.

Plate V. The Lateral Bud of a Stolon Tip Displaying Callus Induction.

Figure 9. Longitudinal section of a lateral bud from Nephrolepis exaltata c.v. 'bostoniensis' displaying the formation of callus tissue (see arrow) from provascular tissue (80 x).

Figure 10. Longitudinal section of a lateral bud from Nephrolepis exaltata c.v. 'scotti' displaying the formation of callus tissue (see arrow) from provascular tissue (80 x).

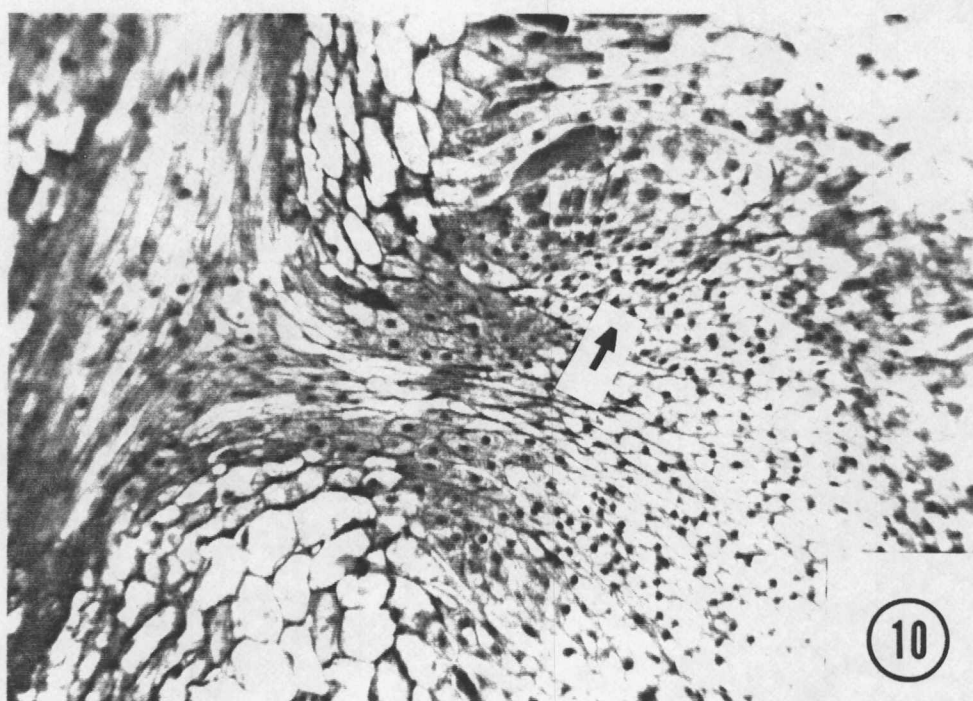
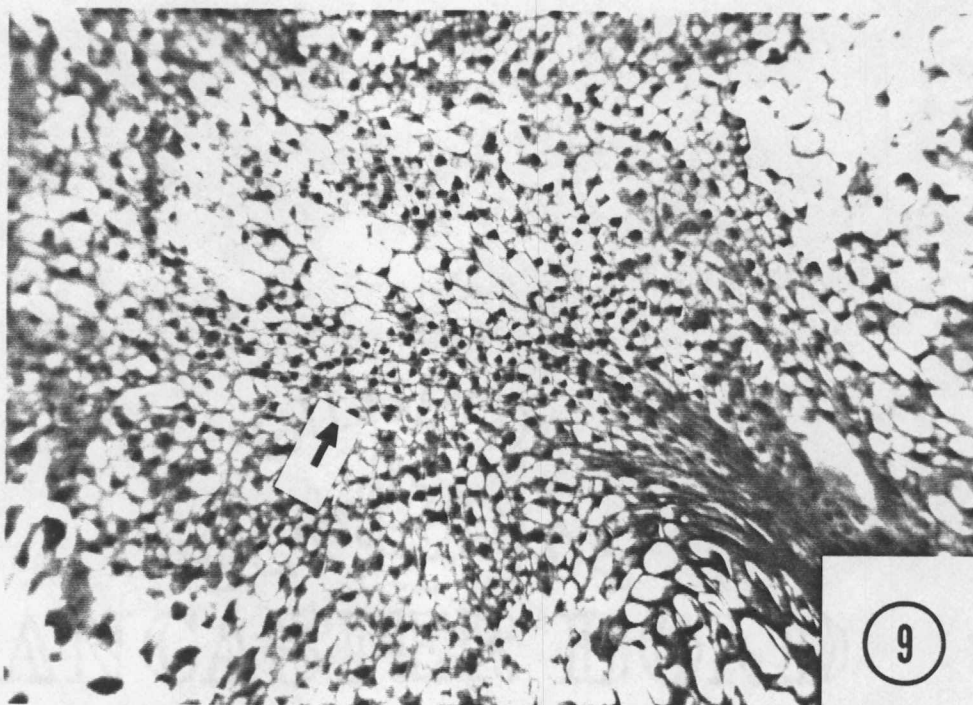


Plate V

A series of experiments with Nephrolepis exaltata c.v. 'bostoniensis' has shown that Murashige Fern Multiplication Medium modified by the addition of 3% sucrose and 0.5 mg/l 2,4-D produced the greatest increase in fresh weight of callus. Table II illustrates also that basal medium containing a hormone concentration of 0.5 mg/l, 2,4-D is significant at the 5% level from 2.0, 1.5, and 1.0 mg/l, 2,4-D treatments. However, treatments 3.0 and 2.5 mg/l 2,4-D differs significantly from 0.5 mg/l and 4.0 mg/l 2,4-D but not from 3.5 mg/l 2,4-D treatments. The 3% sucrose treatment was significantly different from 4% sucrose.

The significance of the data was tested statistically using Duncan's Multiple Range Test. After an F ratio has been observed to be significant, the t-test is often used to determine which specific means differ significantly from each other. However, a more stringent test procedure for making multiple comparisons is the Duncan's Multiple Range Test. In this study of fern callus significant differences did not exist for each pair of treatments. The Duncan's Multiple Range Test creates a partial separation of treatments that were statistically significant at the 5% level and those treatments that were not significantly different at the 5% level. The Duncan Grouping A is significantly different from Duncan Grouping B, but not from Duncan Grouping A,B. The means of the parameter to be evaluated, roots and shoots, are ranked and differences between the ranges of means are compared as being significant if the difference between the mean is larger than the minimum significant difference for that range.

Table II. Significant Differences Among Treatment Means as Determined by Duncan's Multiple Range Test, at the 5 Percent Level for Callus Induction in N. exaltata c.v. 'bostoniensis'.

Concentration	Mean Weight in Grams	Duncan* Grouping
<u>2,4-D in mg/l</u>		
0.5	0.1721	A
2.0	0.1080	B
1.5	0.1057	B
1.0	0.1042	B,C
3.0	0.0851	B,C
2.5	0.0836	B,C
3.5	0.0635	C,D
4.0	0.0482	D
<u>Sucrose in g/l</u>		
3%	0.1152	A
4%	0.1010	A,B
1%	0.0846	B
2%	0.0844	B

*Means with the same letter are not significantly different.

In a second and third series of experiments with N. exaltata c.v. 'dwarf boston' and N. exaltata c.v. 'scotti' several trends were observed (Tables III and IV). Basal medium with 3% sucrose and 0.5 mg/l 2,4-D produced the greatest increase in fresh weight of callus in all cases. In N. exaltata c.v. 'bostoniensis' a concentration of 2.0 mg/l 2,4-D in the medium produced a significant increase in fresh weight (Duncan Group B, Table II). In cultivars N. exaltata c.v. 'scotti' and N. exaltata c.v. 'dwarf boston' medium containing 2.0 mg/l 2,4-D produced an increase in fresh weight that was not significantly different from 3.0 and 2.5 mg/l 2,4-D (Duncan Grouping B and C, Tables III and IV). In all cases the lowest increase in fresh weight was recorded with 3.0, 2.5, 3.5, and 4.0 mg/l 2,4-D concentrations. However, the 4.0 mg/l 2,4-D treatment was not significantly different from 3.5 mg/l 2,4-D treatments with N. exaltata c.v. 'scotti' and N. exaltata c.v. 'bostoniensis' but, was significantly different at the 5 percent level from 3.5 mg/l 2,4-D treatments with N. exaltata c.v. 'dwarf boston'. In all cases the lowest increase in fresh weight was recorded with 4%, 1%, and 2% sucrose concentrations which was significantly different with N. exaltata c.v. 'dwarf boston' and N. exaltata c.v. 'scotti' from 3% sucrose treatments. However, the 3% sucrose treatment was not significantly different from the 4% sucrose treatment with N. exaltata c.v. 'bostoniensis'.

Callus formation from stolon buds took 4 to 6 weeks. Once callus was established in sterile culture, it was possible to

Table III. Significant Differences Among Treatment Means as Determined by Duncan's Multiple Range Test, at the 5 Percent Level for Callus Induction in N. exaltata c.v. 'scotti'.

Concentration	Mean Weight in Grams	Duncan* Grouping
<u>2,4-D in mg/l</u>		
0.5	0.1719	A
1.0	0.1041	B
1.5	0.1036	B
2.0	0.0966	B,C
3.0	0.0822	B,C,D
2.5	0.0752	C,D
3.5	0.0623	D,E
4.0	0.0428	E
<u>Sucrose in g/l</u>		
3%	0.1185	A
4%	0.0920	B
1%	0.0798	B
2%	0.0790	B

*Means with the same letter are not significantly different.

Table IV. Significant Differences Among Treatment Means as Determined by Duncan's Multiple Range Test, at the 5 Percent Level for Callus Induction in N. exaltata c.v. 'dwarf boston'.

Concentration	Mean Weight in Grams	Duncan* Grouping
<u>2,4-D in mg/l</u>		
0.5	0.1810	A
1.0	0.1090	B
1.5	0.1003	B,C
2.0	0.0940	B,C
3.0	0.0839	C
2.5	0.0797	C,D
3.5	0.0599	D
4.0	0.0275	E
<u>Sucrose in g/l</u>		
3%	0.1206	A
4%	0.0888	B
1%	0.0811	B
2%	0.0773	B

*Means with the same letter are not significantly different.

subculture. The subcultured callus covered the surface area of the medium in a 25 mm test tube in 8 weeks. The original callus has been subcultured for over 2 years on Murashige Fern Multiplication Medium modified for callus formation with 0.5 mg/l 2,4-D and 3% sucrose because this provided the greatest fresh weight increase of callus tissue. At other sucrose and hormone concentrations, the callus developed to a less extent. Plate VI, Figure 11, illustrates stolon origin and Figures 12, 13, and 14 demonstrate the typical appearance of Nephrolepis callus derived from the stolons on Murashige Fern Multiplication Medium modified for callus formation with 3% sucrose and differing concentrations of 2,4-D.

Callus Differentiation into Whole Plants from Stolon Callus

The experiments to determine in vitro controlled differentiation of the callus from stolon tips demonstrate a far greater potential for the callus to differentiate into adventitious roots than into shoots and roots. However, shoot and root differentiation of Nephrolepis exaltata c.v. 'bostoniensis', N. exaltata c.v. 'dwarf boston' and N. exaltata c.v. 'scotti' has been successful on media with the hormone combinations of K $5 \times 10^{-7}M$ plus NAA $5 \times 10^{-7}M$, K $10^{-6}M$ plus NAA $10^{-5}M$, and K $10^{-6}M$ plus NAA $5 \times 10^{-7}M$ in the light. Stolon callus cultured in the dark on medium with the same hormone combinations died. An examination of Plate VII, Figures 15 through 19, demonstrates the differentiation of callus into whole plants.

A series of experiments with N. exaltata c.v. 'bostoniensis' callus has shown that the greatest production of shoots with kinetin

Plate VI. Stolon Origin and Callus Induction.

Figure 11. Photograph of stolons which originate from the base of the leaves.

Figure 12. Production of callus at the apical meristem of a stolon from N. exaltata c.v. 'bostoniensis' on basal medium with 3% sucrose and 1 mg/l 2,4-D.

Figure 13. Production of callus at the apical and lateral meristems of a stolon from N. exaltata c.v. 'bostoniensis' on basal medium with 3% sucrose and 2 mg/l 2,4-D.

Figure 14. Production of callus at the apical meristem of a stolon from N. exaltata c.v. 'bostoniensis' on basal medium with 3% sucrose and 3 mg/l 2,4-D.

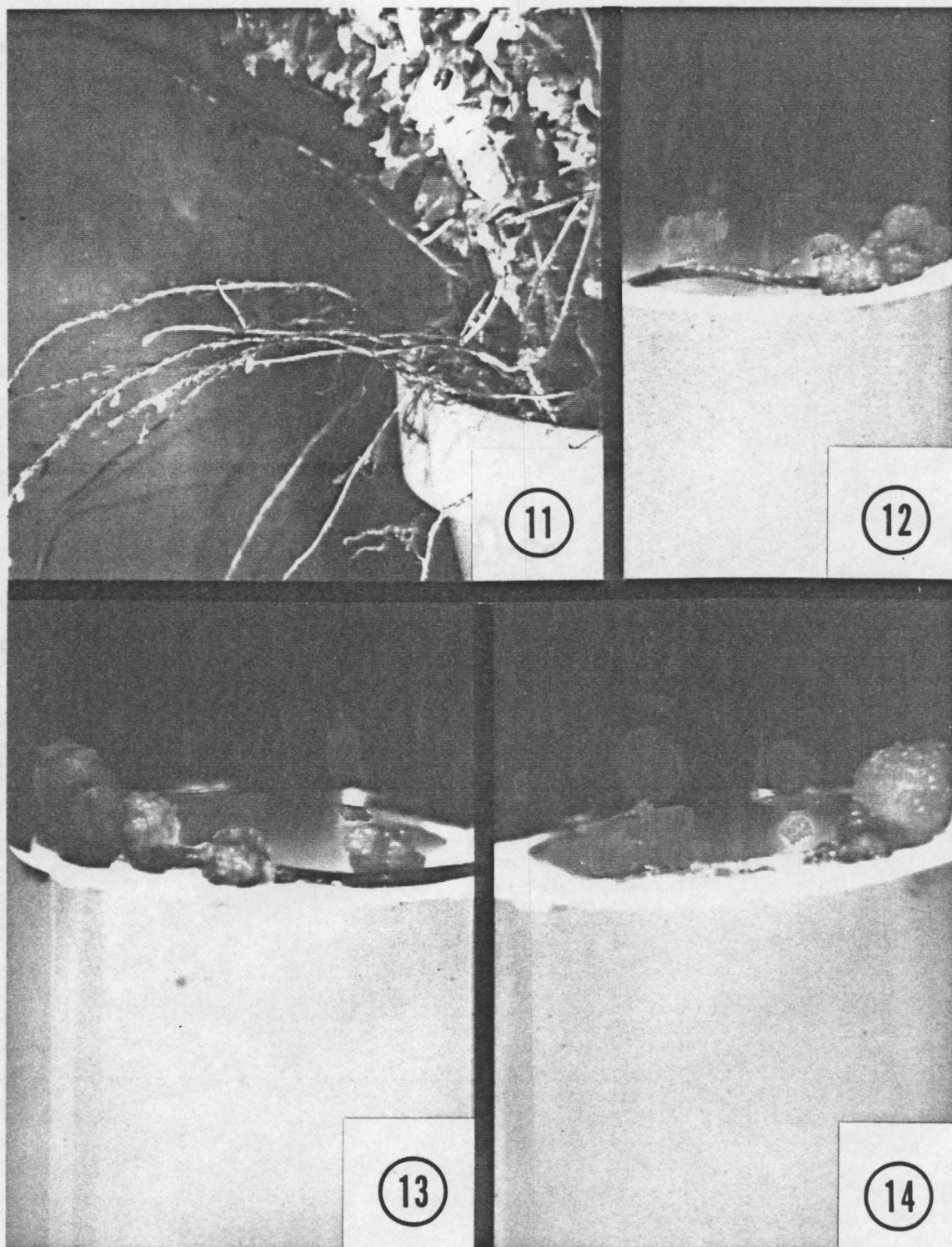


Plate VI

Plate VII. Callus Differentiation.

Figure 15. Typical appearance of callus after subculturing for 10 weeks on basal medium with 3% sucrose and 0.5 mg/l 2,4-D.

Figure 16. Typical appearance of shoot and root differentiation after subculturing callus on basal medium with 10^{-6} M K and 5×10^{-7} NAA at 2 weeks.

Figure 17. Typical appearance of shoot and root differentiation after subculturing callus on basal medium with 5×10^{-7} M K and 5×10^{-7} M NAA at 2 weeks.

Figure 18. Organogenesis at 4 weeks on basal medium with 10^{-6} M K and 5×10^{-7} M NAA. At this stage root and shoot production are clearly distinguishable from the callus tissue.

Figure 19. Organogenesis at 12 weeks showing a plant of sufficient size for re-establishment in soil.

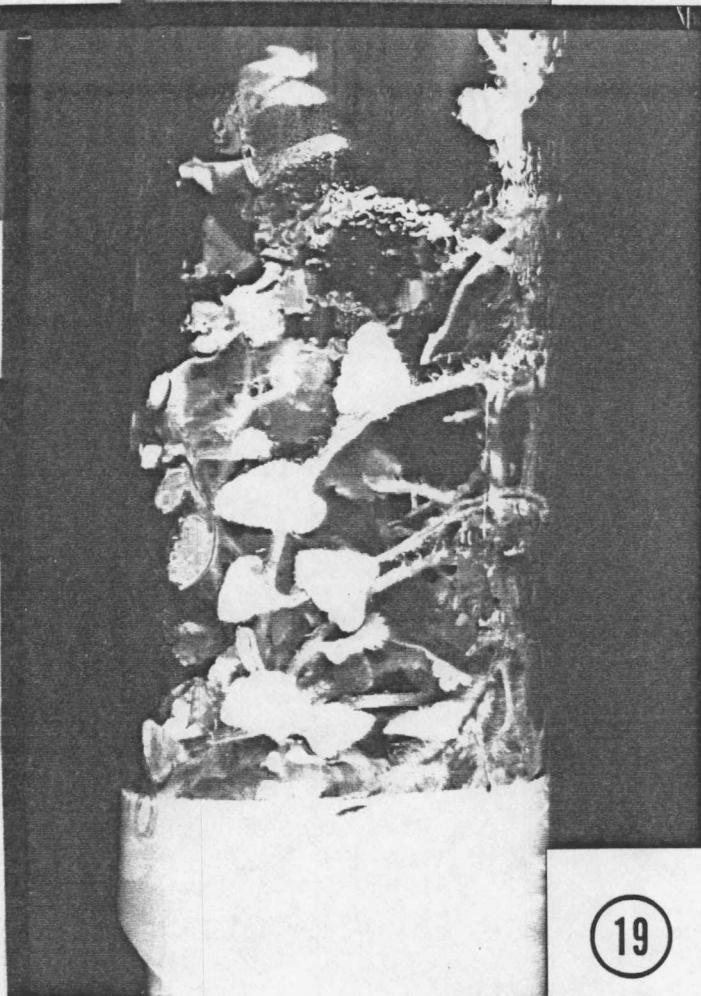
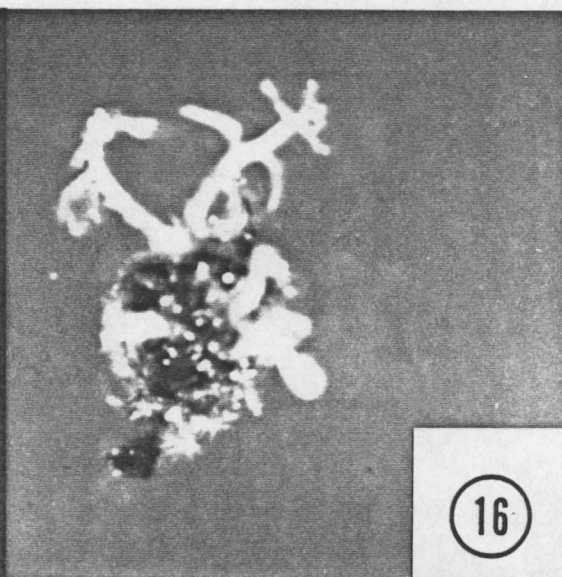
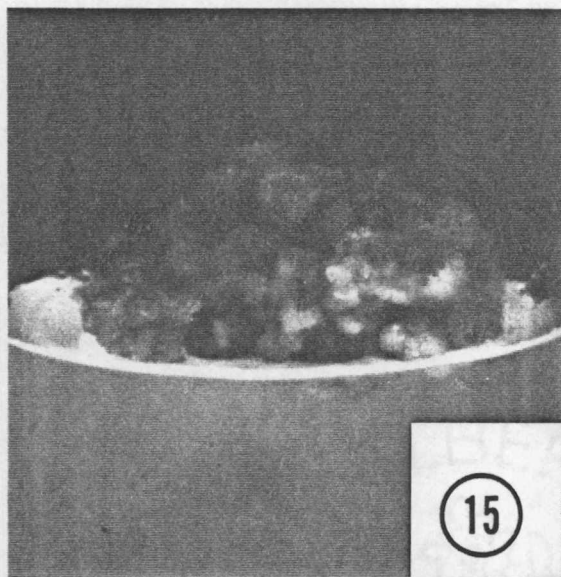


Plate VII

occurred on media with a concentration of 10^{-6}M (Table V) which is significant at the 5 percent level from $5 \times 10^{-7}\text{M}$ and $5 \times 10^{-6}\text{M}$ concentrations. All other concentrations of kinetin failed to produce shoots. The greatest production of shoots with NAA was observed with a concentration of $5 \times 10^{-7}\text{M}$ (Table V) which is significant at the 5 percent level from 10^{-5} and 10^{-6}M concentrations. All other NAA concentrations failed to produce shoots. The greatest production of roots in N. exaltata c.v. 'bostoniensis' occurred on media with kinetin at a concentration of $5 \times 10^{-7}\text{M}$ (Table VI) which was significantly different from 10^{-6}M . Kinetin treatments of 10^{-5}M and $5 \times 10^{-6}\text{M}$ were significantly different from 10^{-6}M and produced a lower mean number of roots, while $5 \times 10^{-5}\text{M}$ failed to produce roots. The NAA concentration which produced the greatest mean number of roots was $5 \times 10^{-5}\text{M}$ (Duncan Grouping A, Table VI) but $5 \times 10^{-5}\text{M}$ was not significantly different at the 5 percent level from $5 \times 10^{-6}\text{M}$ (Duncan Grouping A,B) however, it was significantly different from 10^{-5}M (Duncan Grouping B,C). The NAA concentration which produced the fewest mean number of roots were 10^{-6} and $5 \times 10^{-7}\text{M}$ which were significantly different at the 5 percent level.

In a series of experiments with N. exaltata c.v. 'scotti' callus (Table VII), the greatest production of shoots occurred on media with K in a concentration of 10^{-6}M which was not significantly different from $5 \times 10^{-7}\text{M}$ at the 5 percent level. All other kinetin concentrations failed to produce shoots. The greatest production of shoots with NAA was observed with a concentration

Table V. Significant Differences Among Treatment Means for Shoots as Determined by Duncan's Multiple Range Test at the 5 Percent Level for *N. exaltata* c.v. 'bostoniensis'.

Concentration	Mean Shoots	Duncan* Grouping
<u>Kinetin</u>		
10 ⁻⁶ M	1.83	A
5x10 ⁻⁷ M	0.85	B
5x10 ⁻⁶ M	0.71	B
10 ⁻⁵ M	0.00	C
5x10 ⁻⁵ M	0.00	C
<u>NAA</u>		
5x10 ⁻⁷ M	1.75	A
10 ⁻⁵ M	0.93	B
10 ⁻⁶ M	0.71	B
5x10 ⁻⁶ M	0.00	C
5x10 ⁻⁵ M	0.00	C

*Means with the same letter are not significantly different.

Table VI. Significant Differences Among Treatment Means for Roots as Determined by Duncan's Multiple Range Test at the 5 Percent Level for *N. exaltata* c.v. 'bostoniensis'.

Concentration	Mean Roots	Duncan* Grouping
<u>Kinetin</u>		
5x10 ⁻⁷ M	13.71	A
10 ⁻⁶ M	7.80	B
10 ⁻⁵ M	4.31	C
5x10 ⁻⁶ M	3.71	C
5x10 ⁻⁵ M	0.00	D
<u>NAA</u>		
5x10 ⁻⁵ M	8.60	A
5x10 ⁻⁶ M	7.15	A,B
10 ⁻⁵ M	6.00	B,C
10 ⁻⁶ M	5.10	C
5x10 ⁻⁷ M	2.70	D

*Means with the same letter are not significantly different.

Table VII. Significant Differences Among Treatment Means for Shoots as Determined by Duncan's Multiple Range Test at the 5 Percent Level for N. exaltata c.v. 'scotti'.

Concentration	Mean Shoots	Duncan* Grouping
<u>Kinetin</u>		
10 ⁻⁶ M	0.75	A
5x10 ⁻⁷ M	0.46	A
5x10 ⁻⁶ M	0.00	B
10 ⁻⁵ M	0.00	B
5x10 ⁻⁵ M	0.00	B
<u>NAA</u>		
5x10 ⁻⁷ M	1.00	A
10 ⁻⁵ M	0.21	B
10 ⁻⁶ M	0.00	B
5x10 ⁻⁶ M	0.00	B
5x10 ⁻⁵ M	0.00	B

*Means with the same letter are not significantly different.

of $5 \times 10^{-7}M$ which is significantly different from $10^{-5}M$ at the 5 percent level. All other concentrations of NAA failed to produce shoots. The greatest production of roots in N. exaltata c.v. 'scotti' was observed on media with a kinetin concentration of $10^{-6}M$ (Table VIII) which was significant at the 5 percent level from $5 \times 10^{-7}M$. Kinetin treatments of $10^{-5}M$ and $5 \times 10^{-6}M$ were significantly different from $5 \times 10^{-7}M$ and produced fewer roots, while $5 \times 10^{-5}M$ failed to produce roots. The NAA concentration which produced the greatest mean number of roots was $5 \times 10^{-7}M$ (Table VIII) but was not significantly different from $10^{-6}M$ at the 5 percent level. However, $5 \times 10^{-7}M$ and $10^{-6}M$ NAA concentrations were significantly different (Table VIII) at the 5 percent level from $5 \times 10^{-6}M$, $10^{-5}M$ and $5 \times 10^{-5}M$ NAA concentrations in mean root production.

A series of experiments with N. exaltata c.v. 'dwarf boston' callus has shown that the greatest production of shoots with kinetin occurred on media with a concentration of $10^{-6}M$ (Table IX) which is significant at the 5 percent level from $5 \times 10^{-7}M$ and $5 \times 10^{-6}M$. Kinetin concentrations of $10^{-5}M$ and $5 \times 10^{-5}M$ failed to produce shoots. The NAA concentration which produced the greatest mean number of shoots is $5 \times 10^{-7}M$ which is significantly different at the 5 percent level from $10^{-5}M$ and $10^{-6}M$. All other concentrations of NAA failed to produce shoots.

The greatest production of roots in N. exaltata c.v. 'dwarf boston' occurred on media with kinetin in a concentration of $5 \times 10^{-7}M$ (Table X) which is significant at the 5 percent level from

Table VIII. Significant Differences Among Treatment Means for Roots as Determined by Duncan's Multiple Range Test at the 5 Percent Level for N. exaltata c.v. 'scotti'.

Concentration	Mean Roots	Duncan* Grouping
<u>Kinetin</u>		
10 ⁻⁶ M	19.86	A
5x10 ⁻⁷ M	16.63	B
10 ⁻⁵ M	7.58	C
5x10 ⁻⁶ M	5.25	C
5x10 ⁻⁵ M	0.00	D
<u>NAA</u>		
5x10 ⁻⁷ M	12.50	A
10 ⁻⁶ M	11.73	A
5x10 ⁻⁶	9.13	B
10 ⁻⁵ M	8.88	B
5x10 ⁻⁵ M	7.08	B

*Means with the same letter are not significantly different.

Table IX. Significant Differences Among Treatment Means for Shoots as Determined by Duncan's Multiple Range Test at the 5 Percent Level for N. exaltata c.v. 'dwarf boston'.

Concentration	Mean Shoots	Duncan* Grouping
<u>Kinetin</u>		
10 ⁻⁶ M	1.46	A
5x10 ⁻⁷ M	0.53	B
5x10 ⁻⁶ M	0.20	B,C
10 ⁻⁵ M	0.00	C
5x10 ⁻⁵ M	0.00	C
<u>NAA</u>		
5x10 ⁻⁷ M	1.43	A
10 ⁻⁵ M	0.56	B
10 ⁻⁶ M	0.20	B,C
5x10 ⁻⁶ M	0.00	C
5x10 ⁻⁵ M	0.00	C

*Means with the same letter are not significantly different.

Table X. Significant Differences Among Treatment Means for Roots as Determined by Duncan's Multiple Range Test at the 5 Percent Level for N. exaltata c.v. 'dwarf boston'.

Concentration	Mean Roots	Duncan* Grouping
<u>Kinetin</u>		
5x10 ⁻⁷ M	13.63	A
10 ⁻⁶ M	11.13	B
5x10 ⁻⁶ M	4.71	C
10 ⁻⁵ M	3.26	C
5x10 ⁻⁵ M	0.06	D
<u>NAA</u>		
5x10 ⁻⁵ M	7.85	A
5x10 ⁻⁶ M	6.86	A,B
10 ⁻⁶ M	6.81	A,B
10 ⁻⁵ M	5.86	B
5x10 ⁻⁷ M	5.41	B

*Means with the same letter are not significantly different.

$10^{-6}M$. Kinetin treatments of $5 \times 10^{-6}M$ and $10^{-5}M$ were significantly different from $10^{-6}M$ and produce a lower mean number of roots, while $5 \times 10^{-5}M$ produced the lowest mean number of roots. The NAA concentration which produced the greatest mean number of roots was $5 \times 10^{-5}M$ (Table X) which was not significantly different from $5 \times 10^{-6}M$ and $10^{-6}M$ at the 5 percent level. However, $10^{-5}M$ and $5 \times 10^{-7}M$ NAA concentrations were significantly different at the 5 percent level from $5 \times 10^{-5}M$ NAA but not significantly different from $5 \times 10^{-6}M$ and $10^{-6}M$ NAA.

In an examination of callus differentiation among N. exaltata cultivars the following is demonstrated in Tables XI through XVI. First, differentiation into shoots and roots occurred with callus from all cultivars on media with kinetin $5 \times 10^{-7}M$ plus NAA $5 \times 10^{-7}M$, kinetin $10^{-6}M$ plus NAA $10^{-5}M$, and kinetin 10^{-6} plus NAA $5 \times 10^{-7}M$ (Tables XI through XVI). Only in N. exaltata c.v. 'bostoniensis' was shoot production observed without root production on media with $5 \times 10^{-6}M$ kinetin plus $10^{-6}M$ NAA (Tables XI and XII). In N. exaltata c.v. 'scotti' no shoots were observed with kinetin $5 \times 10^{-6}M$ plus NAA $10^{-6}M$ concentrations (Tables XIII and XIV) which produced shoots with other cultivars. In N. exaltata c.v. 'dwarf boston' both shoot and root production was observed on media with kinetin $5 \times 10^{-6}M$ plus NAA $10^{-6}M$ (Tables XV and XVI).

Second, root production from callus tissue was the greatest from lower ($5 \times 10^{-7}M$) kinetin concentrations with N. exaltata c.v. 'bostoniensis' (Table VI, page 39, and Table XII) and with

Table XI. Mean Number of Shoots from N. exaltata c.v. 'bostoniensis' with Standard Deviation from the Mean.

	Kinetin (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
NAA (M)						
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁷	0.0±0.0	4.2±3.5	4.5±3.9	0.0±0.0	0.0±0.0	0.0±0.0
10 ⁻⁶	0.0±0.0	0.0±0.0	0.0±0.0	3.5±4.3	0.0±0.0	0.0±0.0
5x10 ⁻⁶	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
10 ⁻⁵	0.0±0.0	0.0±0.0	4.6±3.4	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁵	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Table XII. Mean Number of Roots from N. exaltata c.v. 'bostoniensis' with Standard Deviation from the Mean.

NAA (M)	Kinetin (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
0	4.7±2.2	5.1±1.9	4.7±2.1	1.3±0.9	1.0±0.0	0.0±0.0
5x10 ⁻⁷	4.9±2.6	6.4±3.3	5.0±3.0	1.4±2.5	0.5±1.1	0.0±0.0
10 ⁻⁶	10.9±2.1	16.3±7.2	7.5±5.6	0.0±0.0	1.6±2.9	0.0±0.0
5x10 ⁻⁶	11.2±4.3	17.0±5.2	7.5±5.4	7.0±6.1	3.9±4.8	0.0±0.0
10 ⁻⁵	9.3±4.5	13.0±9.4	8.2±4.6	5.7±5.7	2.9±4.1	0.0±0.0
5x10 ⁻⁵	10.1±6.1	25.6±11.8	10.4±5.7	4.4±2.6	12.5±6.6	0.0±0.0

Table XIII. Mean Number of Shoots from N. exaltata c.v. 'scotti' with Standard Deviation from the Mean.

	Kinetin (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
NAA (M)						
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁷	0.0±0.0	2.3±2.3	2.6±3.2	0.0±0.0	0.0±0.0	0.0±0.0
10 ⁻⁶	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁶	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
10 ⁻⁵	0.0±0.0	0.0±0.0	1.0±1.0	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁵	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Table XIV. Mean Number of Roots from N. exaltata c.v. 'scotti' with Standard Deviation from the Mean.

	Kinetin (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
NAA (M)						
0	12.6±5.1	16.2±8.3	16.7±7.1	9.1±4.9	14.1±6.3	0.0±0.0
5x10 ⁻⁷	11.9±3.9	14.7±9.4	22.4±8.3	9.5±11.6	15.7±7.2	0.0±0.0
10 ⁻⁶	13.0±5.8	17.8±5.3	23.0±7.0	4.4±8.6	13.3±7.1	0.0±0.0
5x10 ⁻⁶	15.6±9.3	18.3±5.4	17.5±10.0	6.3±8.3	3.5±4.8	0.0±0.0
10 ⁻⁵	16.2±5.3	12.8±3.6	20.3±7.9	5.9±5.5	5.3±6.1	0.0±0.0
5x10 ⁻⁵	14.7±7.9	19.4±6.3	16.0±8.3	0.0±0.0	0.0±0.0	0.0±0.0

Table XV. Mean Number of Shoots from N. exaltata c.v. 'dwarf boston' with Standard Deviation from the Mean.

	Kinetin (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
NAA (M)						
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁷	0.0±0.0	2.6±2.4	4.5±3.8	0.0±0.0	0.0±0.0	0.0±0.0
10 ⁻⁶	0.0±0.0	0.0±0.0	0.0±0.0	1.0±1.4	0.0±0.0	0.0±0.0
5x10 ⁻⁶	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
10 ⁻⁵	0.0±0.0	0.0±0.0	2.8±3.1	0.0±0.0	0.0±0.0	0.0±0.0
5x10 ⁻⁵	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Table XVI. Mean Number of Roots from N. exaltata c.v. 'dwarf boston' with Standard Deviation from the Mean.

	Kinetin (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
NAA (M)						
0	9.0±6.8	11.3±5.7	7.1±4.7	3.8±2.0	1.9±0.6	0.0±0.0
5x10 ⁻⁷	10.7±3.9	10.2±3.6	9.2±4.8	4.8±4.5	2.4±2.7	0.3±0.6
10 ⁻⁶	13.7±4.8	14.5±5.4	17.5±8.9	0.7±1.7	1.3±2.1	0.0±0.0
5x10 ⁻⁶	12.8±4.5	15.2±5.3	9.6±6.2	6.5±4.7	2.9±2.8	0.0±0.0
10 ⁻⁵	11.3±7.9	12.3±7.0	8.9±4.0	6.5±4.7	1.5±1.9	0.0±0.0
5x10 ⁻⁵	14.6±9.1	15.8±5.2	10.3±4.7	5.0±3.1	8.0±4.0	0.0±0.0

N. exaltata c.v. 'dwarf boston' (Table X, page 44, and Table XVI) and with N. exaltata c.v. 'scotti' root production was greatest with 10^{-6} M kinetin concentrations (Table VIII, page 42, and Table XIV). The greatest root production with NAA concentrations were more difficult to isolate because significant difference did not exist between every pair of treatments (Table VI, page 39; Table VIII, page 42; and Table X, page 44). No roots were observed on media with 5×10^{-5} M concentrations of kinetin regardless of NAA concentrations with N. exaltata c.v. 'bostoniensis' and N. exaltata c.v. 'scotti' (Tables XII and XIV). However, at 5×10^{-5} M kinetin plus 5×10^{-7} M NAA concentrations with N. exaltata c.v. 'dwarf boston' (Table XVI) few roots (0.3 plus or minus 0.6) were observed and at all other 5×10^{-5} M concentrations of kinetin no roots were observed.

Third, the callus of all cultivars failed to product shoots on media with 2ip and IAA. In N. exaltata c.v. 'bostoniensis' did the combination of 10^{-6} M 2ip plus 5×10^{-6} M IAA (Table XVII) produce more roots (24.6 plus or minus 11.3). The greatest mean number of roots was produced with combinations of 5×10^{-7} M 2ip and 10^{-6} M IAA in N. exaltata c.v. 'scotti' (22.5 plus or minus 4.7) and N. exaltata c.v. 'dwarf boston' (23.2 plus or minus 4.3) (Tables XVIII and XIX). In all cultivars no roots were produced at 5×10^{-5} M 2ip regardless of IAA concentrations (Tables XVI, XVIII, and XIX).

Table XVII. Mean Number of Roots from N. exaltata c.v. 'bostoniensis' with Standard Deviation from the Mean.

IAA (M)	Zip (M)				
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	5x10 ⁻⁵
0	13.9±5.6	14.1±9.7	8.0±3.4	7.5±4.6	1.3±0.4
5x10 ⁻⁷	17.8±10.2	19.7±7.9	10.2±4.4	8.4±9.7	1.1±2.7
10 ⁻⁶	19.9±11.7	24.4±4.6	6.2±9.3	9.2±10.6	2.1±3.9
5x10 ⁻⁶	14.1±7.7	12.0±7.3	24.6±11.3	11.6±8.0	1.1±1.3
10 ⁻⁵	11.3±8.2	12.8±10.2	17.0±8.7	8.0±7.2	1.7±1.8
5x10 ⁻⁵	12.7±4.8	19.0±5.1	9.8±5.0	3.7±5.4	0.0±0.0

Table XVIII. Mean Number of Roots from N. exaltata c.v. 'scottii' with Standard Deviation from the Mean.

	2ip (M)					
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	10 ⁻⁵	5x10 ⁻⁵
IAA (m)						
0	12.1±4.5	13.9±7.9	9.7±5.3	3.5±1.2	0.9±0.3	0.0±0.0
5x10 ⁻⁷	13.6±8.1	14.6±10.0	11.0±4.3	7.0±6.0	0.4±1.4	0.0±0.0
10 ⁻⁶	15.6±7.2	22.5±4.7	13.3±7.2	2.0±3.8	5.0±6.7	0.0±0.0
5x10 ⁻⁶	13.9±9.2	16.8±9.0	18.5±7.5	2.8±3.4	2.9±5.9	0.0±0.0
10 ⁻⁵	11.1±8.3	19.1±5.2	16.0±5.1	7.2±2.8	7.2±10.1	0.0±0.0
5x10 ⁻⁵	13.7±7.4	22.5±7.1	10.5±5.4	3.0±2.9	0.0±0.0	0.0±0.0

Table XIX. Mean Number of Roots from N. exaltata c.v. 'dwarf boston' with Standard Deviation from the Mean.

IAA (M)	2ip (M)				
	0	5x10 ⁻⁷	10 ⁻⁶	5x10 ⁻⁶	5x10 ⁻⁵
0	12.1±8.0	12.4±4.8	11.8±5.0	8.0±3.6	1.6±3.0
5x10 ⁻⁷	13.5±6.5	16.6±7.1	12.5±4.1	9.5±7.2	2.1±3.6
10 ⁻⁶	15.3±9.7	23.2±4.3	10.4±6.1	6.7±5.4	2.8±4.4
5x10 ⁻⁶	14.3±8.7	14.2±9.9	15.0±6.2	5.0±4.1	2.5±4.1
10 ⁻⁵	11.4±4.9	14.1±9.4	16.2±3.3	6.5±4.6	4.9±5.7
5x10 ⁻⁵	12.3±5.6	17.5±7.3	12.3±4.9	4.2±3.7	0.1±0.5
					0.0±0.0

Fourth, after the first subculture of N. exaltata callus, the ability to produce shoots was lost. However, the capacity to form roots is still present after multiple subcultures of the callus.

Callus Induction from Cell Suspension Cultures

Four to five grams of Nephrolepis callus was used to initiate a suspension culture for each cultivar. Due to the soft texture of Nephrolepis callus found in all cultivars, agitation in a liquid medium produced suspensions of free cells and multicellular aggregates. Variation in the morphological appearance of free cells was observed in all cultivars of N. exaltata. The cell morphologies were helical (30-40x140-180 microns), bifurcated (40x200 microns), spherical (10-50 microns), comma or "C" shaped (30-50x100-150 microns), gourd-shaped (30-40x80-140 microns) and elongated cells (20-30x200 microns) (Plates VIII and IX). In addition to the above mentioned shapes, cells appeared with combinations of morphologies; for example, Plate IX, Figure 25, displays an elongate morphology with a slight fork or bifurcation.

After 3 weeks a 10 ml aliquot was subcultured under aseptic conditions to fresh medium by the method of Davey, Fowler, and Street (1971). Observations of cell number and packed cell volume by the method of Henshaw et al. (1966) demonstrated the presence of a viable callus suspension culture for Nephrolepis exaltata c.v. 'bostoniensis' and the cultivars N. exaltata c.v. 'scotti'

Plate VIII. Callus Cells in Suspension Culture.

Figure 20. A single cell with helical morphology from a callus suspension culture of Nephrolepis exaltata c.v. 'bostoniensis', the bar represents a distance of 10 microns (200 x).

Figure 21. A single cell with a bifurcation from a callus suspension culture of N. exaltata c.v. 'dwarf boston' displaying a different morphology from the above figure (200 x).

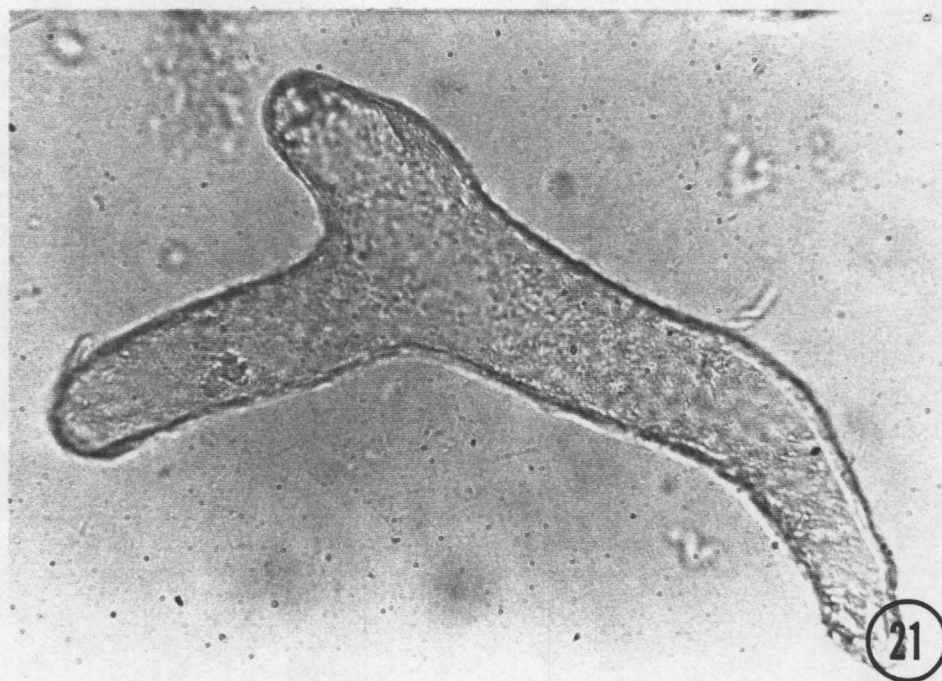
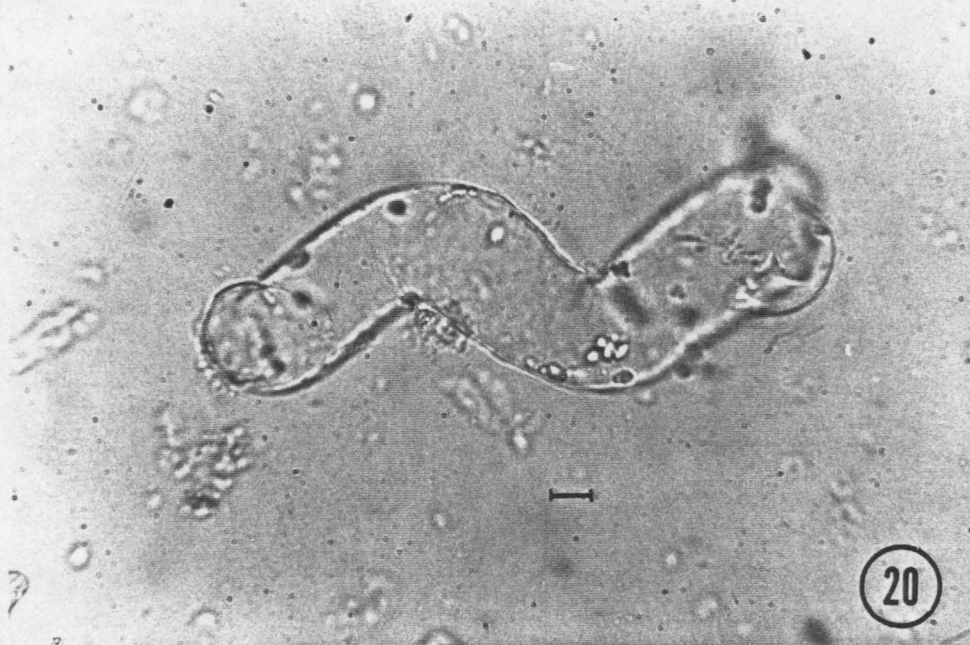


Plate IX. Callus Cells in Suspension Culture.

Figure 22. A spherical cell which was typical in callus suspension cultures. This cell was from a suspension culture of N. exaltata c.v. 'scotti' (80 x).

Figure 23. A comma or "C" shaped cell was frequently observed in callus suspension cultures. This cell was from a callus suspension culture of N. exaltata c.v. 'bostoniensis' (80 x).

Figure 24. Multicellular aggregates were observed among the single cells in suspension cultures. A gourd-shaped morphology was regularly observed in culture. These cells were from a suspension culture of N. exaltata c.v. 'bostoniensis' (80 x).

Figure 25. Cells of elongate morphology were also present in callus cultures. This cell was from a callus suspension culture of N. exaltata c.v. 'dwarf boston' (80 x).

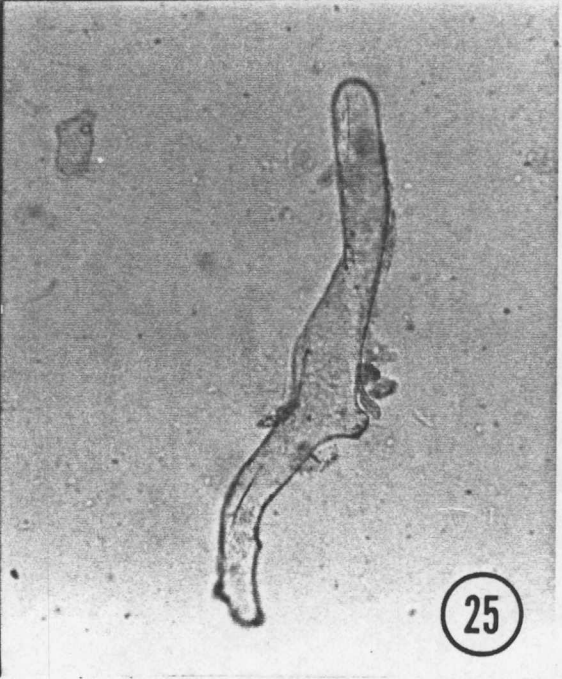
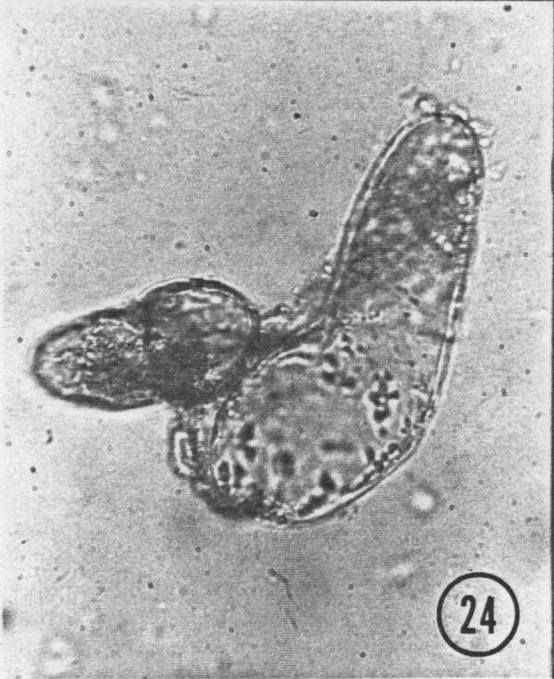
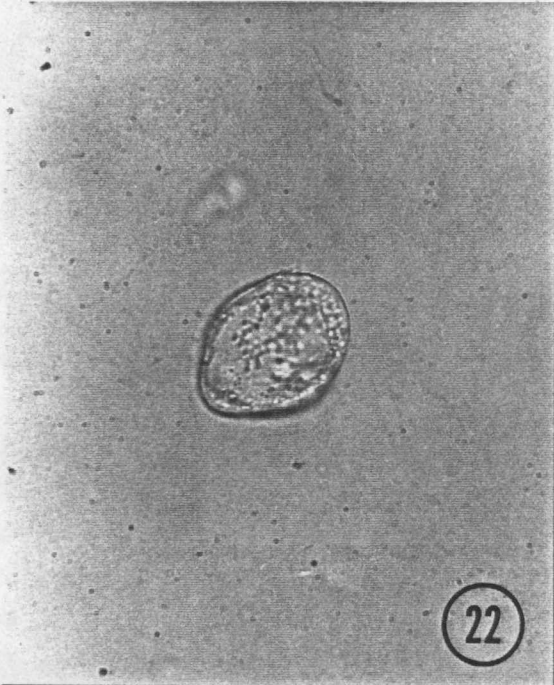


Plate IX

and N. exaltata c.v. 'dwarf boston'. The growth pattern indicates that after a short lag phase, there occurs synchronous growth due to large numbers of single cells undergoing division simultaneously. This growth reaches a stationary phase in about 18-20 days (Plates X and XI). An examination of the packed cell volume demonstrates that it continues to increase during the stationary phase as a result of cell volume expansion (Plate XI).

To initiate the growth of isolated single cells into callus clones for future organogenesis, the Bergmann Technique (1960) was employed. Tables XX, XXI, and XXII demonstrate that higher cell densities per plate produced higher callus clone plating efficiencies. In all three cases, 2,000 callus cells per Petri plate produced the greatest increase in callus clones. In recording cell divisions from suspension cultures, it was observed that some cells would divide five or six times and then stop dividing, while other cells that did not begin to divide in the first 6 days after a week began to divide and established callus clones. This phenomena was also observed by Bergmann (1960) with Nicotiana tabacum and Phaesolus vulgaris. After the callus clones were established on the Bergmann plates (Plate XII, Figures 28, 29, and 30), they were transferred to Murashige Fern Multiplication Medium modified with $5 \times 10^{-7}M$ NAA and $10^{-6}M$ K for organogenesis. Plate XIII, Figures 34 through 37, demonstrates the appearance of callus clones which have undergone organogenesis and formed whole plants.

Plate X. Growth of Cell Numbers from Cell Suspension Cultures.

Figure 26. This graph displays the growth of cell numbers for N. exaltata c.v. 'bostoniensis' (open circle), N. exaltata c.v. 'scotti' (paired squares), and N. exaltata 'dwarf boston' (i) over a period of 27 days.

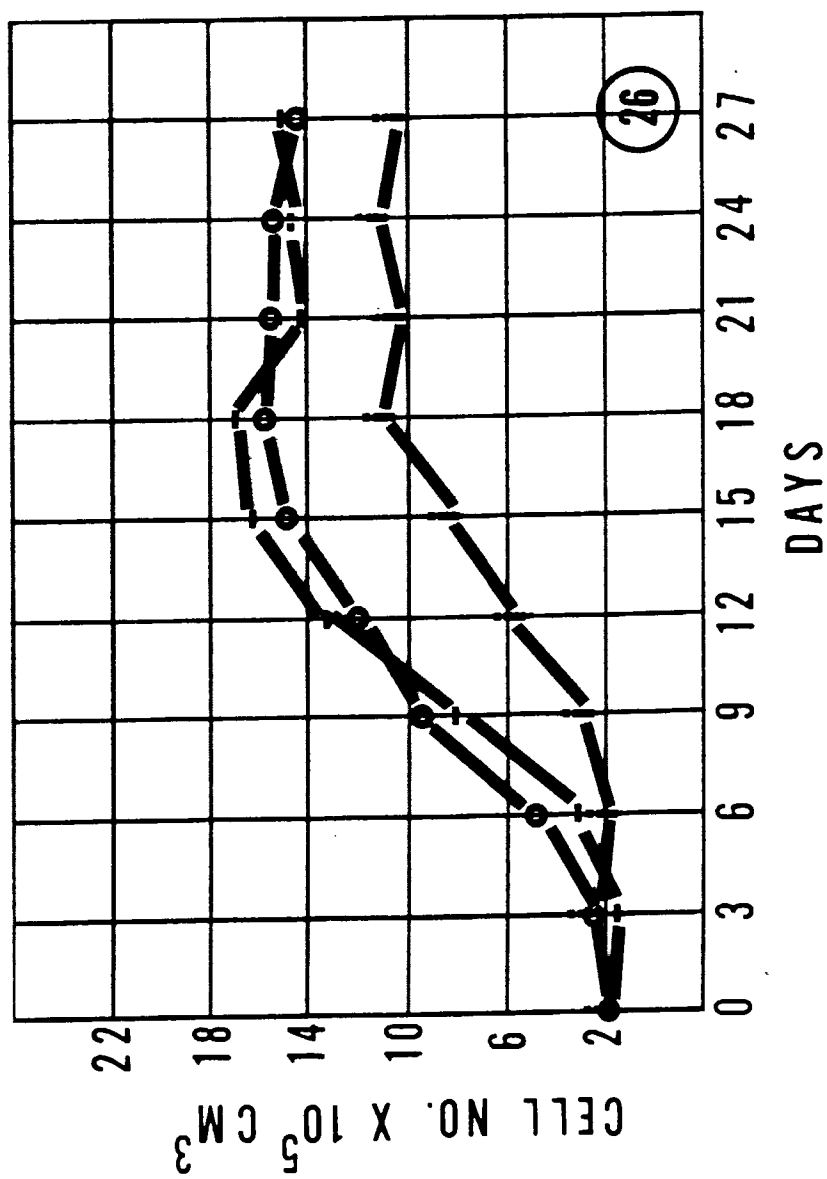


Plate X

Plate XI. Growth of Packed Cell Volume from Cell Suspension Cultures.

Figure 27. This graph displays the growth of packed cell volume for N. exaltata c.v. 'bostoniensis' (open circle), N. exaltata c.v. 'scotti' (paired squares), and N. exaltata c.v. 'dwarf boston' (i) over a period of 27 days.

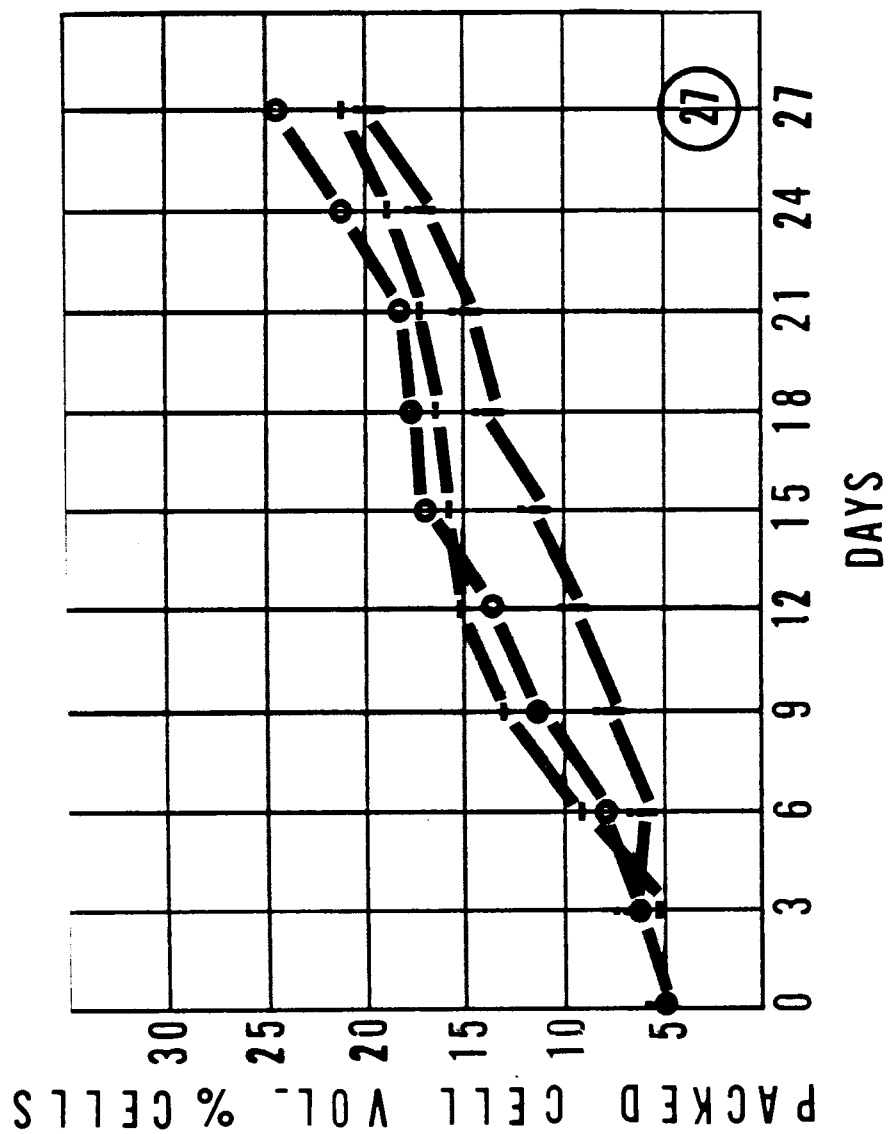


Plate XI

Table XX. Multiplication of Plated Cells of N. exaltata c.v. 'bostoniensis' at Different Cell Densities.

Cell Density Per Plate	Days After Plating	No. of Cells Which Divided to Form*				Sum	Percent
		2-3 Cells	4-15 Cells	16-32 Cells	Clone		
125	0	7				7	5.6
	6	15	4			19	15.2
	12	26	9			35	28.0
	24	43	14	1		57	45.6
	30	51	21	2		74	59.2
250	0	21				21	8.4
	6	57	17			74	29.6
	12	72	19	6		97	38.8
	24	103	31	8		142	56.8
	30	121	39	10		170	68.0
500	0	29				29	5.8
	6	71	23			94	18.8
	12	132	23	8		174	34.8
	24	152	42	12		206	41.2
	30	209	63	17	3	293	58.4
1000	0	54				54	5.4
	6	137	36			173	17.3
	12	283	73			356	35.6
	24	406	91	58		555	55.5
	30	539	187	93	5	824	82.4
2000	0	97				97	4.8
	6	221	43			264	13.2
	12	391	79	20		490	24.5
	24	643	142	73	3	861	43.0
	30	1141	407	128	12	1688	84.4

*Average of six plates, a cell mass with a diameter of 3 mm was called a clone.

Table XXI. Multiplication of Plated Cells of N. exaltata c.v. 'scotti' at Different Cell Densities.

Cell Density Per Plate	Days After Plating	No. of Cells Which Divided to Form*				Sum	Percent
		2-3 Cells	4-15 Cells	16-32 Cells	Clone		
125	0	7				7	5.6
	6	24	7			31	24.8
	12	57	17			74	59.2
	24	63	19	1		83	66.4
	30	65	21	3		89	71.2
250	0	31				31	12.4
	6	47	19			66	26.4
	12	68	37			105	42.0
	24	87	48	5		140	56.0
	30	117	48	7		172	68.8
500	0	32				32	6.4
	6	79	30			109	21.8
	12	142	47	7		196	39.2
	24	163	71	16		250	50.0
	30	207	103	35	2	347	69.4
1000	0	47				47	4.7
	6	92	29			121	12.1
	12	172	41	2		215	21.5
	24	268	107	16		391	39.1
	30	383	164	39	5	591	59.1
2000	0	107				107	5.3
	6	213	67			280	14.0
	12	471	79	22		572	28.6
	24	749	193	51	1	994	49.7
	30	1293	236	98	8	1635	81.7

*Average of six plates, a cell mass with a diameter of 3 mm was called a clone.

Table XXII. Multiplication of Plated Cells of N. exaltata c.v. 'dwarf boston' at Different Cell Densities.

Cell Density Per Plate	Days After Plating	No. of Cells Which Divided to Form*				Sum	Percent
		2-3 Cells	4-15 Cells	16-32 Cells	Clone		
125	0	5				5	4.0
	6	27	2			29	23.2
	12	46	9			55	44.0
	24	57	11	3		71	56.8
	30	61	19	5		85	68.0
250	0	27				27	10.8
	6	41	21			62	24.8
	12	63	29	2		94	37.6
	24	74	33	6		113	45.2
	30	94	41	8		143	57.2
500	0	33				33	6.6
	6	59	29			88	17.6
	12	153	43	4		200	40.0
	24	195	67	11		273	54.6
	30	269	79	15	2	365	73.0
1000	0	61				61	6.1
	6	153	42			195	19.5
	12	317	85			402	40.2
	24	438	126	41		605	60.5
	30	521	178	89	6	794	79.4
2000	0	81				81	4.0
	6	258	51			309	15.4
	12	467	81	32		580	29.0
	24	755	216	51	4	1026	51.3
	30	1206	383	111	11	1711	85.5

*Average of six plates, a cell mass with a diameter of 3 mm was called a clone.

Plate XII. The Bergmann Plating Technique.

Figure 28. This photograph displays the typical appearance of a Bergmann plate with 500 cells per plate, the arrow demonstrates the position of the callus clone.

Figure 29. A photograph of a Bergmann plate with 1000 cells per plate which has produced several clones (arrow).

Figure 30. A photograph of 2000 cells per plate, which throughout the study produced more callus clones, displaying several clones (arrow).

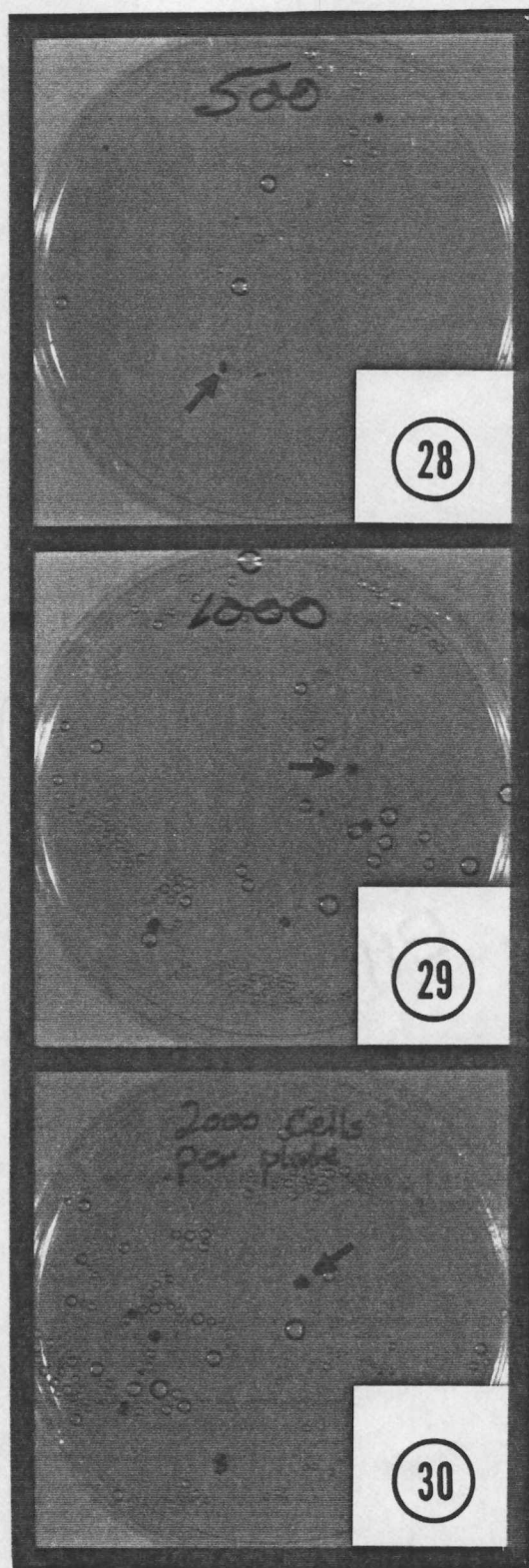


Plate XII

Plate XIII. The Organogenesis of Callus Clones.

Figure 31. This photograph displays the appearance of N. exaltata c.v. 'scotti' that has been grown from a callus clone taken from a Bergmann plate.

Figure 32. This photograph displays the appearance of N. exaltata c.v. 'dwarf boston' that has been grown from a callus clone taken from a Bergmann plate.

Figure 33. This photograph displays a double shoot of N. exaltata c.v. 'bostoniensis' that has been grown from a callus clone taken from a Bergmann plate.

Figure 34. This photograph is of a callus clone of N. exaltata c.v. 'bostoniensis' at 12 weeks on morphogenic medium. This plant is of sufficient size for re-establishment in soil.

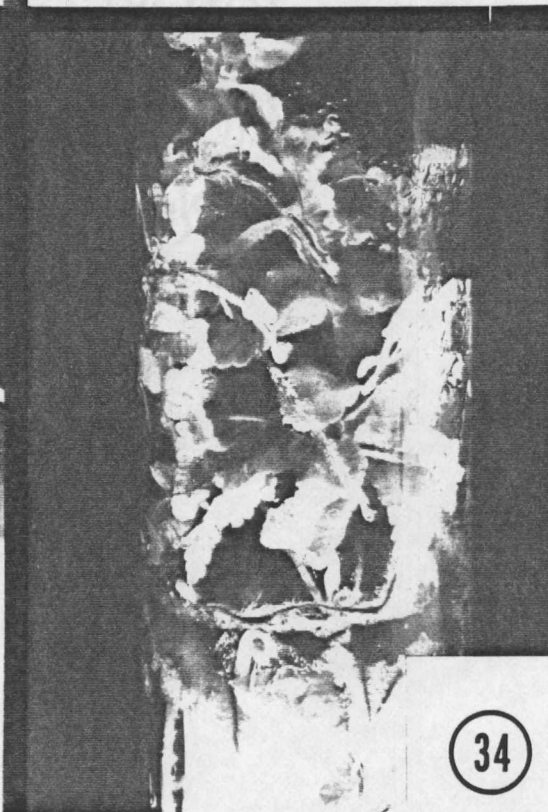
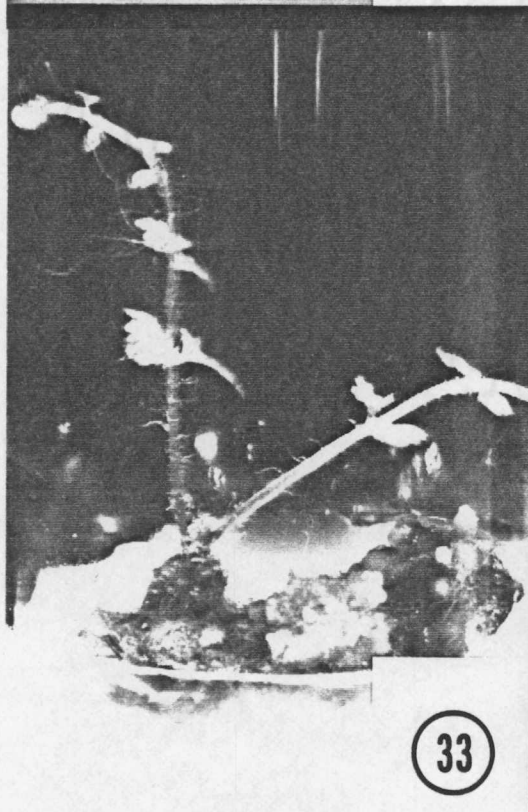
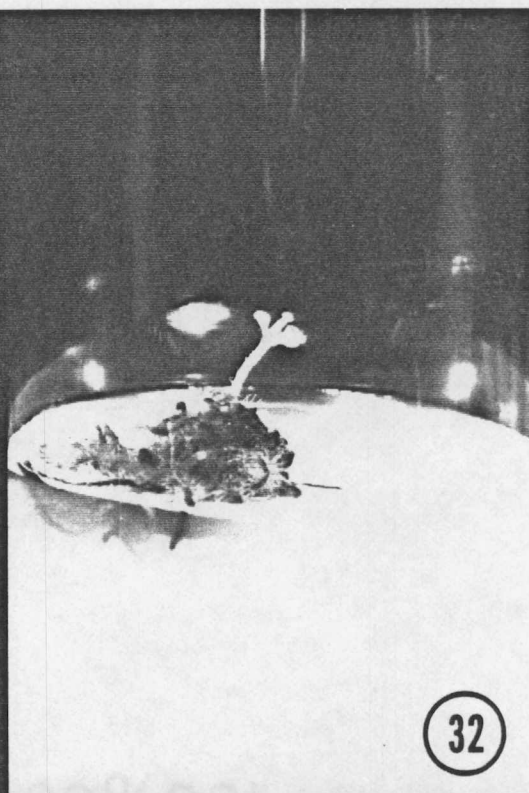
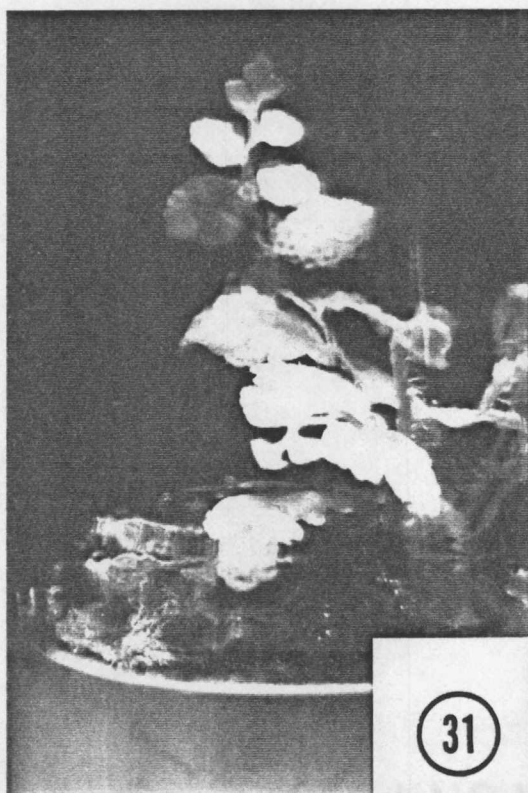


Plate XIII

IV. DISCUSSION

This research has demonstrated that Boston Fern and two of its cultivars are capable of totipotency. Stolon tips are capable of producing callus. The callus in turn is capable of differentiating into whole plants. It is interesting to note that these phenomena occur in Boston Fern and two of its cultivars. This may not be too surprising in light of the fact that all three cultivars share the same morphology of corresponding areas of leaf, rhizome, and stolon. Moreover, they have the same morphology as Nephrolepis biserrata (Sw.) Schott as shown by Espagnac (1973). The anatomical study also demonstrated that the origin of callus in all three cultivars was provascular tissue in terminal and lateral buds of stolon tips. This is a new discovery in that researchers have induced callus in gametophytes, leaf tissues, buds, upright stems, and roots, but not in stolon tissue. The synthetic auxin, 2,4-D, will induce callus in Pteris cretica (Bristow, 1962), Pteris vittata (Kato, 1963), and Cyclosorus dentatus (Mehra and Palta, 1972). Nephrolepis callus was induced by the same growth substance (2,4-D) as Pteris vittata, Pteris cretica, and Cyclosorus dentatus. The combination of kinetin plus 2,4-D will induce callus in Ophioglossum petiolatum (Peterson, 1967). The gametophytes of Pteridium aquilinum (Steeves and Sussex 1952), Osmunda cinnamomea (Morel, 1951), Adiantum pedatum (Morel and Wetmore, 1956), and Lycopodium obscurum (DeMaggio, 1964) require no callus inducing

hormones and will spontaneously form callus. Wound reactions will induce callus in buds of Ophioglossum petiolatum (Peterson, 1967) and in gametophytes of Pteridium aquilinum (Partanen, Sussex, and Steeves, 1955). Kinetin is required to induce callus in Marsilea vestita (Laetsch and Briggs, 1961).

Yeoman (1970) states, "Excised plant tissue can be divided into four main groups with regard to their callusing properties. All of the groups require a carbon source, usually a sugar, and a mineral salts mixture." The groups are as follows: (1) the first require only an auxin, (2) the second group of tissue requires both an auxin and cytokinin, (3) the third group does not require additives (callus formed spontaneously or with wounding), and (4) the fourth group of tissues requires only cytokinin. Yeoman's remarks were addressed to callus induction in angiosperms, but clearly pteridophytes demonstrate callusing trends which fall into all four main groups.

Callus induction in Nephrolepis cultivars occurred only when the tissues were cultured on a revised medium containing both sucrose and the auxin 2,4-D. Nephrolepis callus did not develop in the absence of sucrose. This effect has also been observed with Pteris cretica (Bristow, 1962) and Cyclosorus dentatus (Mehra and Palta, 1972). Gautheret (1955) observed the phenomena of habituated tissue in which certain strains of callus require exogenous hormones for growth but acquired the capacity to grow as callus after transfer to medium without exogenous hormones.

Habituated callus tissue was not observed in N. exaltata callus cultures. When callus was subcultured to basal medium free of 2,4-D, the process of root formation was initiated and callus growth ceased.

Callus derived from stolon buds of N. exaltata cultivars was differentiated into entire sporophytes with complete shoot and root systems. The process of differentiation required the presence of both an auxin (NAA) and a cytokinin (kinetin) at specific concentrations. However, with a different auxin (IAA) and cytokinin (2ip) only root production was observed.

Kohlenback (1977) reported the following generalizations concerning plant organogenesis from cell and tissue culture at the First International Congress of Medicinal Plant Research in Munich, Germany.

1. Removal of auxin. After a preculture with auxin, its omission often leads to root formation (Reinert, 1959 with carrot; Thomas and Street, 1970 with Atropa belladonna).
2. Ratio of auxin to cytokinin. A high ratio of auxin relative to cytokinin favors root formation whereas an opposite one or an increased level of cytokinin, respectively a reduced level of auxin favors shoot buds (Durand et al., 1973, with Petunia; Lang and Kohlenback, 1975, with Macleaya cordata; Skoog, 1971, with tobacco).
3. Absolute concentration of phytohormones. In Lycopersicon leaf callus root initiation occurred with 2 mg/l IAA plus 2 mg/l kinetin. Shoot formation only takes place with 4 mg/l IAA plus 4 mg/l kinetin (Padmanabhan et al., 1974).
4. Nature of the auxins and cytokinins. In Asparagus calluses derived from protoplasts shoots can arise with benzyladenine (BA) in combination with IAA or NAA but not with media containing zeatin or 2,4-D (Bui Dang Ha, 1974).

Induction of shoots and roots in N. exaltata callus tissue is dependent upon both the ratio of auxin to cytokinin and the nature of the auxins and cytokinins. However, the classic cytokinin to auxin ratios do not induce the production of shoots and roots from all tissues to the same degree. N. exaltata callus demonstrated a greater potential for differentiation into roots than into roots and shoots. The low percentage of shoots formed may be due to an inhibition of the differentiation process (as opposed to initiation) suggested by Walker, Wendeln, and Jaworski (1979) where shoots and roots were initiated with specific hormone treatments, but differentiation did not occur until hormones were lacking in the medium. Narayanswamy (1977) observed, "rooting occurs more often than other forms of regeneration, irrespective of the source of the callus and is sometimes too random to define its condition."

With progressive subculture, fern callus tissue lost the ability to form shoots after the first transfer but retained a root forming capacity. Syono (1965) observed different phases in carrot root callus morphogenetic potential with continuing subculture until all organ forming potential was lost. Syono designated his phases as follows: Phase I, both roots and buds are formed; Phase II, at second subculture only buds formed; Phase III, at third subculture both roots and buds could be formed with different culture conditions, but the frequency was low; Phase IV, at the fourth subculture all organ forming potential was lost.

In the N. exaltata callus differentiation study a clearly separated phase was observed with the loss of shoot formation after the first subculture. Modifications to the medium did not restore shoot formation (Phase III) in N. exaltata callus. The N. exaltata callus differentiation study does reinforce the experimental evidence of Syono, that with progressive subculture morphogenetic potential of callus is altered. The complete loss of organ formation (Phase IV) has not occurred even after seven subcultures. Roots are still produced from callus of N. exaltata cultivars and may be produced for numerous subcultures. Barba and Nickell (1969) observed in sugarcane callus that shoot formation may decrease with age, but organogenesis of rooting persists for years.

Gautheret (1966) has suggested that callus cultures lose the ability to synthesize specific metabolites required for organogenesis and thus fail to produce organs. Such chemical compounds are still being researched and identified. One such compound has been observed by Howard et al. (1977) who reported the presence of a soybean agglutinin which serves as a mitogen for soybean callus cells and may have functions as a growth regulator. This aspect needs further investigation.

An alternative hypothesis for the loss of organogenesis with subculturing has been advanced by Murashige and Nakand (1967) who observed conditions of ploidy change and aneuploidy in tobacco callus cultures incapable of organogenesis. Torrey (1958, 1961) contends the accumulation of aneuploid and polyploid cells occurs

when tissues are repeatedly subcultured. Mutations present themselves with the subculture of callus tissue and organogenesis occurs in cells with nuclei free of chromosomal mutations thus a decline in organogenesis is observed.

Attempts to examine N. exaltata cultivars ($2n=82$) and callus tissues for aneuploidy were unsuccessful (Dr. Hickok, personal communication) due to large chromosome number. Attempts to classify callus cells as polyploids did not reveal any clearly distinguishable polyploid cells. This is a problem for future research in fern callus differentiation.

The soft texture of Nephrolepis exaltata callus allowed for the production of suspension cultures with gentle agitation. The morphology of the single cells in suspension culture displayed great variation. This phenomena was also observed by Halperin (1969) who stated, "The majority of cells in any culture have gross cytological features similar to thin-walled ground parenchyma of the intact plant, but the resemblance does not include a regularity of shape. A variety of shapes, many quite bizarre, occurs due to random yielding of wall areas and irregular orientation of cell plates." The callus suspension cultures of Nephrolepis were viable and all cultivars increased in both cell numbers and cell volume. The growth of cells in suspension culture demonstrate the presence of distinguishable growth phases. A short lag phase in which cells are prepared for division, was followed by a phase of rapid cell division as explained by Thomas and Davey (1975) in their work.

A stationary phase was reached in about 18-20 days in all cultivars in which the cell volume increased but the cell numbers remain relatively constant as shown by Henshaw et al. (1966) in his work.

When a suspension culture of low cell numbers is inoculated on Bergmann's plates, they may grow poorly or fail to grow at all. Halperin (1969) believes the failure of low cell numbers to divide may be due to excessive leakage of metabolites into the medium. Wareing and Phillips (1981) hypothesize that isolated single cells are leaky due to their large surface areas exposed to the liquid medium and these cells tend to lose substances required for division into the surrounding medium. Thus, in order to induce growth from single cells of suspension cultures, one must employ the following: (1) greatly increase the single cell concentration per Bergmann plate or, (2) employ a conditioned medium containing the "leaked" special nutrient factors necessary for cell division. Stuart and Street (1969) observed that conditioning of the medium provided essential complexes of amino acids that were necessary for cell division.

The results presented in this research have demonstrated the techniques for callus induction, proliferation and differentiation of Nephrolepis exaltata c.v. 'bostoniensis', N. exaltata c.v. 'scotti' and N. exaltata c.v. 'dwarf boston'. Thus, the morphogenic totipotency of pteridophytes has been achieved with the production of whole sporophytes from callus tissue and single cells of Nephrolepis.

Even though differentiation has been achieved from callus derived from stolon tips and from cell suspension, additional studies are needed in achieving differentiation from protoplast cultures. Protoplast fusion studies would be valuable not only in basic whole plant differentiation studies but in fern hybridization studies.

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