This is to certify that the

dissertation entitled

PROPAGATION OF ACER RUBRUM L. 'RED SUNSET' IN VITRO

presented by

Kent James Welsh

has been accepted towards fulfillment
of the requirements for

DOCTOR OF PHILOSOPHY degree in HORTICULTURE

K. C. Hinkle
Major professor

Date July 24, 1986

PROPAGATION OF *ACER RUBRUM* L. 'RED SUNSET' *IN VITRO*

By

Kent James Welsh

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1985

ABSTRACT

PROPAGATION OF ACER RUBRUM L. 'RED SUNSET' IN VITRO

By

Kent James Welsh

A protocol as developed for propagating Acer rubrum L. 'Red Sunset' in vitro. A preconditioning treatment which consisted of subculturing shoot tip or node segment explants, 3-4 cm, at 3-4 day intervals on LS basal medium or with 1.0 mgl^{-1} BA or 2ip was required to establish aseptic, actively growing cultures. The most effective cytokinin for stimulating axillary shoot proliferation was 6-benzylaminopurine (BA) with 5.9 shoots/explant occurring at 5.0 mgl^{-1}. Shoot proliferation was most consistent following decapitation. Incorporation of 100 mgl^{-1} adenine sulfate in LS + 10.0 mgl^{-1} 2ip multiplication medium also stimulated axillary shoot proliferation. Rapid growth in vitro led to the development of vitreous shoots. Vitrification was effectively controlled in 'Red Sunset' shoot tip cultures by harvesting shoots before damage occurred and by culturing explants in 60 ml bottles rather than 25 x 150 mm culture tubes. 'Red Sunset' shoot tips rooted readily on LS basal medium or on LS + IBA, NAA or IAA during a 14 day culture period and were easily acclimated to greenhouse conditions. Plants recovered from culture were uniform phenotypically although their leaves were morphologically different from mature, flowering plants.

To Laura Lee, Jennifer, Melanie and Erica

ACKNOWLEDGEMENTS

I would like to thank Dr. Kenneth C. Sink for his assistance in this research program and in editing this dissertation. I would also like to thank Dr. Harold Davidson for his assistance with this project. Thanks are also given to Dr. Stanley G. Howell, Dr. Lowell C. Ewart, Dr. Harry H. Murakishi and Dr. James W. Hanover for their help in the preparation of this dissertation. Thanks and appreciation also go to my wife for her support and patience while the research was being conducted and during the time spent preparing this dissertation. Thanks also go to my parents for the upbringing and encouragement the have given me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Grafting has been used for centuries to propagate selected plant types; many of which are difficult or impossible to propagate by other classical asexual or sexual methods. Physiological disorders sometimes encountered with grafted plants, however, prompt the testing of new propagation techniques.

Red Maple, Acer rubrum L., is a prime example of this situation. As a species, A. rubrum grows over an extended ecological range covering much of the eastern United States and southeastern Canada (55, 106, 107). Cultivars have been selected from this diverse population for fall coloration, habit, and hardiness. These cultivated types make attractive shade trees and have found use in street plantings, parks and in the home landscape (55).

Until recently these cultivars were strictly propagated by budding; with red maple seedlings as the understock (81). Budding red maple is a relatively simple procedure but graft incompatibility often develops. Losses of 50% the first year after budding and an additional 10-20% the second year have been reported (85). The cause of this incompatibility is not known but the genetic diversity of the stock and scion is quite probably involved (20, 55).

Propagating these cultivars by cuttage is one obvious way to bypass the incompatibility problem. And, there are a number of reports (17, 20, 30, 31, 58, 81, 85, 97, 113) which describe methods for rooting node or stem tip cuttings of red maple. Propagation by cuttings, however, is

limited by: 1) the size of the stock block required, 2) seasonal propagation period and 3) the extended period of time required to increase new selections. Micropropagation techniques may be useful in overcoming these limitations (57, 69, 100).

To determine the feasibility of propagating red maple *in vitro* four objectives were established in this research study: 1) to develop procedures for initiating aseptic cultures using shoot tips from mature, flowering trees, 2) to develop an *in vitro* shoot proliferation system, 3) to determine procedures for *in vitro* rooting and 4) to evaluate the uniformity of plants propagated *in vitro*.

LITERATURE REVIEW

In the 80 years since Haberlandt published his work on the culture of isolated plant cells, tissue culture has grown from an enlightened idea to a scientific reality. The isolation and purification of indoleacetic acid (IAA) by Kogl and associates in 1934 (104, 116) and the discovery of 6-furfurylaminopurine (kinetin) (71,72) played a large role in the emergence of this field. The real impetus for tissue culture, however, came with the realization that it was possible to control growth and organization *in vitro* by manipulating the ratio of auxin(s) and cytokinin(s) in the culture media (94). The pioneering work of Skoog and Miller (94) led to the development of many tissue culture techniques including plant propagation.

Woody species have generally been considered to be more difficult to propagate *in vitro* than herbaceous species (100), however, evidence is accumulating which refutes this supposition. Abbott (1, 2) suggested that this inconsistency was due to a lack of effort and inadequate research in this area. The rapid increase in the number of references dealing with *in vitro* propagation of woody species supports this arguement (Table 1).

Winton (119) stated that commercial propagation of woody species using tissue culture techniques was hampered by low frequency shoot proliferation and by the use of seedling explants. While this is still true to a certain extent, some success in developing commercially feasible micropropagation protocols has been made in the *Betulaceae*

(69), Ericaceae (63, 100) and Rosaceae (22,99). And, the number of articles dealing with in vitro propagation of mature plants and cultivars, including ornamentals has dramatically increased in the last five years.

Developing a micropropagation protocol is a matter of trial and error (1) and is dependent on genotype/environment interactions. While there are some general guidelines to follow such as using cytokinin(s) or cytokinin/auxin combinations to stimulate shoot development and auxin(s) to enhance rooting it is not always clear how a particular species will respond. In culture it is possible to closely control and monitor the environment but the genotype or genetic makeup of an individual normally cannot be predictably altered. Therefore, it is important to understand how the genotype responds in a particular environment and how to manipulate the environment to obtain the optimum response.

Table 1. Woody plant species which have been propagated *in vitro*.

Species	Explant	Explant Source	Reference
Aceraceae			
Acer platanoides 'Crimson Sentry'	Shoot tips	Greenhouse grown trees	22
A. rubrum seedlings 'Red Sunset'	Shoot tips	2 yr-old seedlings Nursery grown trees	115
Betulaceae			
Alnus glutinosa	Lateral buds	Seedlings	35
Betula pendula	Stem internodes	Seedlings	45
	Catkins	Mature trees	98
B. platyphylla azechuanica	Shoot tips Node segments	Seedlings	69
B. verrucosa	Shoot tips Stem internodes Leaves	2-20 yr-old trees	19
Ericaceae			
Kalmia latifolia	Shoot tips	3 yr-old seedlings	63
Rhododendron sp.	Stem segments		57
#704-792-1450	Shoot tips		100
Exbury Azalea DH23, DH28	Shoot tips		33
Vaccinium ashei seedlings 'Becky Blue' 'Bluebelle'	Shoot tips	Seedlings Mature plants	65,66

Table 1 (cont'd)

Species	Explant	Explant Source	Reference
V. corymbosum	Node segments	Mature plants	25
Moraceae			
Morus alba *yanagiba*	Leaves	Seedlings	80
Nyctaginaceae			
Bougainvillea *glabra* 'Magnifica'	Shoot apices	Mature plants	21
	Shoot apices	Field grown plants	86
Proteaceae			
Grevillea sp. 'Robyn Gordon'	Axillary buds	Greenhouse grown plants	36
'Crosbie Morrison'	Node segments	Field grown plants	
G. rosmarinifolia	Node segments	1 yr-old seedlings	10
Rosaceae			
Chaenomeles *japonica*	Shoot tips	Field grown plants	79
Cotoneaster *dammeri* 'Mooncreeper'	Shoot tips	Field grown plants	79
Crataegus *brachyacantha*	Shoot tips	Field grown plants	79
C. x mordenensis 'Toba'	Shoot tips	Field grown plants	79
Malus			
Rootstocks			
Antonovka KA313	Stem segments	Greenhouse grown plants	22

Table 1 (cont'd)

Species	Explant	Explant Source	Reference
EMLA 7 EMLA 9 EMLA 27 MAC 9 M 7	Shoot tips		47
M26	Shoot tips		49
Cultivars			
'Stark Jumbo'	Stem segments	Greenhouse grown plants	22
'Granny Smith'		Greenhouse grown plants	93
'Jonathan' 'Delicious'			
M. domestica 'Golden Delicious	Shoot tips	Field grown trees	64
M. sylvestris 'Cox's Orange Pippin'	Shoot tips	Seedlings Greenhouse grown plants	3
'McIntosh	Meristems	Seedlings	110
M. sieboldii zumi 'Calocarpa'	Shoot tips	Greenhouse grown plants	87
M. sp. 'Almey'	Shoot tips	Greenhouse grown plants	87,88
'Dainty' 'Golden Hornet'	Shoot tips	Field grown plants	79
'Hopa'	Shoot tips	Greenhouse grown plants	87
M. x purpurea 'Eleyi'	Shoot tips	Greenhouse grown plants	87

Table 1 (cont'd)

Species	Explant	Explant Source	Reference
Potentilla fruticosa			
'Coronation Triumph'	Shoot tips	Field grown plants	79
'Sutter's Gold'			
Prunus			
Rootstocks			
Cherry			
Prunus avium x *pseudocrasus*			
'Colt'	Stem segments	Greenhouse grown plants	22
P. avium F 12/1	Shoot tips	1 yr-old greenhouse grown plants	48
P. mahaleb x Mazzard 14 (*P. avium*)	Stem segments	Greenhouse grown plants	22
Peach			
P. persica			
'Nemaguard'	Shoot tips		73
Plum			
P. cerasifera			
'Myrobalan'	Shoot tips	1 yr-old seedlings	38
P. insititia			
'Pixy'	Shoot tips	Greenhouse grown plants	22
		1 yr-old plants	48
'St. Julien X'	Stem segments	Greenhouse grown plants	22

Table 1 (cont'd)

Species	Explant	Explant Source	Reference
Cultivars and Species			
P. amygdalus	Dormant buds	Seedlings	70,103
P. amygdalus x *persica*	Dormant buds		103
P. avium			
'Bing'	Dormant buds	Field grown plants	96
'Black Tartarian			
'Royal Ann'			
'Sam'			
P. cerasifera			
'Atropurpurea'	Shoot tips		34
'Newport'	Stem segments	Greenhouse grown plants	22
'Thundercloud'	Shoot tips	Field grown plants	79
P. cisterna	Shoot apices	Field grown plants	59
P. salicina			
'Calita'	Shoot tips		83
P. tomentosa	Shoot tips	Field grown plants	79
Pyracantha coccinea			
'Lalandei	Shoot tips	Field grown plants	79
Pyrus			
Rootstocks			
Old Home x Farmingdale 51	Stem segments	Greenhouse grown plants	22

Table 1 (cont'd)

Species	Explant	Explant Source	Reference
Cultivars			
P. communis			
'Bartlett'	Shoot apices	Field grown plants	60
'Seckel'	Shoot tips	Greenhouse grown plants	88
Rosa canina	Shoot tips Node segments Lateral buds	Greenhouse grown plants	54
R. damascena	Shoot tips Node segments Lateral buds	Greenhouse grown plants	54
R. hybrida			
'Forever Yours'	Shoot tips	Greenhouse grown plants	92
'Fragrant Cloud' 'Garnet Yellow'	Buds	Greenhouse or field grown plants	26
'Improved Blaze'	Shoot tips	Aseptic stock cultures	41,46
'Kings Ransom' 'Lili Marlene' 'Parade' 'Pauls Lemon Parade' 'Plentiful'	Buds	Greenhouse or field grown plants	26
R. hybrida			
'Bridal Pink' 'Tropicana'	Shoot tips Node segments Lateral buds	Greenhouse grown plants	54
Spirea x *bumalda*			
'Anthony Waterer'	Shoot apices	Field grown plants	59
'Frobelii'	Shoot tips	Field grown plants	79

Table 1 (cont'd)

Species	Explant	Explant Source	Reference
Salicaceae			
Populus alba	Terminal and axillary buds Shoot tips		23
P. alba x glandulosa	Terminal and axillary buds Shoot tips		23
P. alba x tremula	Terminal and axillary buds Shoot tips		23
P. canescens	Cambium Shoot tips		18
P. euroamericana	Cambium Shoot tips		18
P. nigra typica	Cambium Shoot tips		18
P. tremula	Cambium Shoot tips		18
	Terminal and axillary buds Shoot tips		23
P. tremuloides	Stem segments		118
	Terminal and axillary buds Shoot tips		
Saxifragaceae			
Ribes inebrians	Shoot tips	Greenhouse grown plants	120

Genotype Effects

Differential responses between individuals *in vitro* can partially be attributed to their genotypes (54). This statement may refer broadly to interfamily differences or to distantly related members of the same family and may also refer to varietal, cultivar, or individual plant differences. These different morphogenetic responses may indicate different growth regulator requirements or a difference in morphogenetic potential.

Certain groups of individuals seem to respond more readily in culture than others. This is true of the *Rosaceae*; Table 1. includes 24 references and covers 28 *Rosaceous* species. The large amount of research which has been done with this family is due to the horticultural and ornamental value of its members and to the ease with which they can be manipulated in culture. Members of the *Rosaceae* are generally more responsive to 6-benzylaminopurine (BA) or BA/auxin combinations than to other cytokinins or cytokinin/auxin combinations.

The *Ericaceae* is another family which responds readily in culture. Members of this family which can be propagated *in vitro* are: azaleas, *Rhododendrons*, mountain laurel and blueberries. Shoot proliferation is stimulated in these species by relatively high levels (5-15 mgl^{-1}) of 6(γ-γ-dimethylallylamino) purine (2ip) (6, 25, 33, 57, 63, 65, 66, 100).

In comparison there are few published reports of micropropagation of *Acer* species. This may indicate that members of this family are more difficult to manipulate *in vitro* than other species. Cheng (22) placed A. *platanoides* 'Crimson Sentry' with a group of *Rosaceous* species which were less responsive than others being examined. Welsh et al. (115) reported that it was possible to stimulate axillary shoot development on

shoot tips of A. rubrum seedlings and 'Red Sunset', however, the results were inconsistent.

Differential responses have also been observed between members of the same family. Norton and Boe (79) compared the morphogenetic potentials of 8 Rosaceous species and observed a range of responses from Malus 'Dainty' with 4 shoots/explant to Spirea 'Frobelii' with 38 shoots/explant. The optimum BA concentrations for these 8 species varied from 0.1 to 2.5 mgl^{-1}. In a separate study (59), Spirea bumalda out yielded Prunus cisterna by more than 10:1 shoots/explant.

Closely related species also vary in their morphogenetic responses. Differences in optimum growth regulator requirement and shoot number were observed between Prunus tomentosa and P. cerasifera with 2.5 mgl^{-1} BA and 15 $\pm$ 1.4 shoots/explant or 0.1 mgl^{-1} BA and 6 $\pm$ 0.8 shoots/explant respectively (79). Shoot number also varied between Crataegus brachyacantha with 10 $\pm$ 2.2 shoots/explant and the cv 'Toba' with 5 $\pm$ 1.4 shoots/explant, however, the growth regulator requirement was the same.

In a comparison of 4 crabapple cultivars, Singha (87) observed that the responses of Malus sp. 'Almey'; and 'Hopa' were similar with 7.3 or 7.1 shoots/explant on 1.0 mgl^{-1} BA while M. sieboldii var. zumi 'Calocarpa' and M. x purpurea 'Eleyi' responded more readily to 2.0 mgl^{-1} BA with 5.9 or 5.7 shoots/explant, respectively.

Gorst et al. (36) observed variation in nutritional and hormonal requirements and mode of proliferation between two Grevillea hybrids.

Inter and intra specific differences in the number of shoots/explant have been observed between Rosa canina. R. damascena, R. hybrida 'tropicana' and 'Bridal Pink' (54). Similar differences have been reported with other Rosa cultivars (26, 41).

Intra specific differences have also been reported between 4 blackberry cultivars (14).

These reports make it clear that the response of a plant selection to *in vitro* conditions is strongly influenced by its genotype. Similar observations have been made with *Solanaceous* species (89, 114).

Propagation Stages

Murashige (75) described 3 stages for *in vitro* propagation of plants: Stage 1: Culture inititation; Stage 2: Multiplication and Stage 3: Rooting and transfer of plants from culture. While these stages have been recently reviewed (7, 44), they will also be considered here because it is important to understand what occurs in each stage and how they are inter-related.

The introduction of woody plant material to the *in vitro* culture environment is often complicated by surface or systemic contaminants (36, 67, 74, 91) and phenolic secretion from excised tissues (14, 67, 74, 115). Both of these problems must be overcome to establish aseptic, actively growing cultures.

Some tissues are more difficult to surface sterilize than others (91). Fungi and bacteria become trapped in crevices in the bark of explants or at leaf axils and are difficult to dislodge (74). Pubescent shoots are especially difficult to sterilize because the hairs trap contaminants and prevent penetration of the surface sterilants (91).

The use of explants from field grown woody plants represents a significant problem in establishing aseptic cultures (36, 74, 91). Gorst et al. (36) initiated cultures of two *Grevillea* hybrids and found that field grown material was more difficult to surface sterilize than

greenhouse grown plants. They suggested that stock plants should be kept under glass. Strode et al. (100) had to remove Rhododendrons from humid field conditions and place them in a dry, air conditioned room to obtain clean explants. Earlier, Abbott and Whiteley (3) found that it was not necessary to surface sterilize apical primordia excised from greenhouse grown seedlings and adult flowering trees of Malus sylvestris 'Cox's Orange Pippin'. Certainly, where possible, the use of greenhouse grown stock plants holds an advantage over field grown plants for culture initation. However, in many instances it may be necessary to use field grown material. Under these conditions, the plant material and/or the surface sterilization procedures must be manipulated to insure establishment of aseptic cultures.

The time of year also has an effect on the level of surface contaminants. Skirvin (91) found that peach cuttings taken in the spring while dormant and forced in a warm room were more easily sterilized than those collected later in the year. Others (60, 64, 80) have used the same procedure to obtain clean explants.

A variety of treatments are sometimes used prior to surface sterilization to reduce levels of surface contaminants. Jones and Hopgood (48) placed Prunus shoot tips under running water for 50 minutes. This procedure reduced the level of contaminants and made them more accessible to sterilizing agents (44). Dilute detergent solutions, which act as wetting agents, are also commonly used as pretreatments (6, 21, 33, 109). This treatment is especially helpful with pubescent tissues (44). An ethanol dip, normally 70%, may also be employed as a pretreatment (14, 21, 23, 63). Ethanol acts as a wetting agent (7, 11). Broome and Zimmerman (14) used a 70% ethanol plus 1% Tween 20, 15 second dip before placing blackberry explants in a bleach solution. Chaturvedi

et al. (21) used a sequential pretreatment, with a soak in 5% detergent followed by a quick dip in 95% ethanol. Soaks in fungicides are also used (14). The pretreatments may be used singly or sequentially depending on the nature of the tissue and the level of surface contamination.

Aqueous diluted, commercial bleach, 5.25% sodium hypochlorite, is commonly used as a surface sterilant. It acts by the release of chlorine (11). Other chemicals used as surface sterilants include: calcium hypochlorite (33, 36), 0.01% mercury chloride (19, 21), 70% ethanol (45) and 80% isopropanol (96). The concentration and length of treatment varies, depending upon the species, type of tissue, season of year and explant source. In some cases, solutions are agitated (7, 33) or placed under vacuum (7, 33, 91) to encourage better penetration of the surface sterilants.

Antibiotics and fungicides have sometimes been included in culture media to eliminate surface and systemic contaminants, but with limited effectiveness and they are sometimes phytotoxic (15).

Secretion of polyphenols from cut surfaces or damaged tissues is another serious problem encountered in initiating cultures of many woody species. Phenols are released from explants as a result of wounding or injury caused by surface sterilization agents(14). The brown coloration of damaged tissues and surrounding media is due to the oxidation of polyphenols (43, 67).

McComb (67) suggested that phenols could be controlled by adding polyvinylpyrilodone (PVP) to the culture medium or by using antioxidants. Welsh et al. (115) however, reported that PVP, ascorbic acid and L-cysteine were not effective in controlling phenolic secretion from Acer rubrum shoot tips. Broome and Zimmerman (14) reported that

soaking blackberry explants in ascorbic acid, citric acid or cysteine HC1 following surface sterilization resulted in further injury.

Control of phenolic secretion has been reported by transferring tissues to fresh media every one or two days (14, 67). Cheng (22) used a "preconditioning treatment" to control phenolic secretion. The treatment consisted of culturing explants on basal medium for one week followed by dissection into 3-10 mm segments and transfer to appropriate media. This treatment was used to eliminate contaminated or injured tissues and to select actively growing explants. This procedure was also found to be effective by Welsh et al. (115).

Culturing primary explants of blackberry (14) or blueberry (65) in liquid media on a rotating wheel has also been shown to be effective in controlling blackening of explants.

Induction and maintenance of rapid shoot multiplication are the main objectives of Stage 2 (75). In some instances this may occur during the initial culture period although it may take up to two years to acclimate some woody species (68). Abbott and Whiteley (3) reported that shoot tips from mature 'Cox's Orange Pippin' trees required eight weeks culture before shoot proliferation began. Subculture of these *in vitro* induced shoots led to a higher proliferation rate. Similar increases in shoot proliferation have been observed with Rubus (14), *Prunus* (48), *Betula* (69) and *Rosa* (40) cultures. Hasegawa (41) suggested that the increased responsiveness of *Rosa hybrida* shoot tips was due to a change in the physiological state of the explants.

Murashige (77) and Winton (119) suggested that it may be necessary to return explants to a juvenile state before regeneration of woody species can be achieved. Juvenile tissues are generally thought to have a higher morphogenetic potential in culture than tissues from mature

plants (14, 67, 99). This may be partially due to the physical condition of the explants. Stokes (99) observed that it was easier to initiate cultures of woody species during early stages of active growth, before the tissues had begun to lignify. Thus, returning mature explants to an active growth phase may stimulate shoot proliferation.

Rejuvenation has been reported in cultures of Betula (45), Malus (93), Vaccinium (65, 66) and Vitis (90). Symptoms of rejuvenation include: changes in morphological characters (65, 90, 93), increased growth and shoot proliferation rates (65, 66) and improved root formation (65, 66 93). Skene and Barlass (90) observed that Vitis shoots grown from mature 'Cabernet Sauvignore' explants more closely resembled seedlings than the parent. Lyrene (65) described two types of shoots which arose from mature Vaccinium shoot tips. The first type had mature characteristics with large leaves and thick stems while the second type exhibited juvenile, filamentous stems and small leaves. The in vitro induced "juvenile" shoots proliferated vigorously and were easier to root than adult shoots or seedlings.

This reversion to a juvenile state may merely be a feature of growth in vitro (9, 45) although similar observations have been made with plants which were severly pruned (24). Trippi (108) stated that asexual reproduction results in rejuvenation, and Stokes (99) reported that juvenile characteristics could be induced in coniferous species by grafting shoots from mature trees on to seedlings.

Methods for propagating plants in vitro can be divided into two main catagories: 1) those which take advantage of the organized state of the primary explant and 2) those which induce an unorganized state (1, 67). The pathway taken is somewhat dependent on the inherent ability of the plant species to respond (7) although it may be possible

to stimulate even the most recalcitrant species if enough time and energy is spent (1, 119).

Stimulating axillary shoot development from shoot tips or node segments is the most commonly used propagation procedure (1). Multiplication rates are lowest using this propagation method (77) although even modest shoot proliferation can produce large numbers of plants in one year. This is due to a decreased generation time (2). This method has the least risk of inducing aberrant plants (1, 2).

With some species, it has been possible to stimulate adventitious shoot development. Since performed buds are not essential, a larger range of explants can be used. Shoot proliferation rates are generally greater using adventitious systems compared to axillary systems, however, the chance of obtaining abnormal plants is increased (77).

Callus and cell suspension cultures have the greatest potential for mass cloning of plants (77, 119). This is possible at least theoretically, because each cell carries the genetic information necessary to develop into an intact plant. Plants could be recovered via organogenesis or somatic embryogenesis (119). These two types of unorganized cell cultures grow rapidly. Abbott (1, 2) reported that 20-fold increases in cell populations were possible in cell suspensions over a 10 day period. These types of cultures can be maintained indefinitely by routine subculture. Use of callus or cell suspension cultures for propagating woody plants, however, has been limited due to difficulties in obtaining shoot regeneration (1, 2, 76). These types of cultures are also subject to producing higher numbers of aberrant plants, especially from older cultures (1, 2, 105).

Cytokinin(s) either singly or in combination with auxin(s) are normally required to stimulate shoot development from shoot tip or node

segment explants. The mode of action is still a matter of speculation (112), however, it is clear that cytokinin(s) interact with auxin(s) in the release of axillary buds from apical dominance (42, 84, 112, 117).

In intact plants, axillary buds can be released from apical dominance by removal of the terminal bud (12, 41, 112). Decapitation has also been used *in vitro* to stimulate axillary shoot development in *Bougainvillea* (86), *Prunus* (96), *Pyrus* (60), *Rhododendron* (100), *Rosa* (41, 54) and *Rubus* (14) cultures. Snir (96) reported that decapitating cherry shoots increased the shoot multiplication rate 2-3 times.

The three most commonly used cytokinins are 6-furfurylaminopurine (kinetin or Kin), 6-benzylaminopurine (BA) and 6-(γ-γ -dimethylallylamino) purine (2ip). Of these cytokinins, 2ip is the most active while Kin and BA have approximately the same activity (75, 76). Cytokinin specificity, however, and not activity appears to be the most important factor in stimulating shoot proliferation.

BA is the most effective of these cytokinins in stimulating shoot proliferation *in vitro* and elicits responses over a wide range of genotypes including *Betula* (19), *Bougainvillea* (86), *Malus* (64), *Prunus* (103), *Rosa* (40, 41) and *Vitis* (90).

Although 2ip has the highest biological activity, it is only capable of stimulating responses over a narrow range of woody genotypes. This may indicate that its mode of action and recognition site at the cellular level is more specific than that of BA. In the *Ericaceae*, 2ip is the most effective cytokinin in stimulating shoot proliferation (6, 25, 33, 57, 63, 65). It is normally used singly at 5 to 15 mgl^{-1} although Anderson (6) and Strode et al. (100) reported that it was necessary to include indoleacetic acid (IAA).

With other woody species, 2ip has little effect. Lundergan and

Janick (64) compared shoot proliferation rates of Malus cultures following treatment with BA, Kin or 2ip and found that 2ip was the least effective while BA was the most effective. They did report, however, that shoots which developed on media with BA were stunted, while those on 2ip appeared normal. In Rosa cultures Hasegawa (41) found that 2ip could substitute for BA with only a slight decrease in shoot proliferation, however, it was necessary to use 2ip at 10 times the optimum BA concentration.

Kinetin is the least effective of these cytokinins in stimulating shoot proliferation in woody species (86).

Two reponses which appear to be common with all cytokinins are that shoot proliferation increases with increasing concentration, while shoot elongation decreases (25, 39, 59, 87, 109). Production of normal shoots is the main concern. Lane (59) reported that shoot proliferation in Pyrus cultures was the greatest at 2.0 mgl^{-1} BA, however, the shoots were compact and often fasciated. Normal shoots developed at a lower level of BA. The same type of response was observed in Vitis cultures following BA treatment (39).

Vitrification is a term (27) used to describe deleterious changes in the morphology and anatomy of explants (62) as a result of environmental factors in vitro (122). Symptoms of this phenomenon include: the development of translucent or succulent, water soaked or brittle shoots and leaves (5, 27, 33, 37, 62, 101, 122) and tissue breakdown and exudation (63). The leaves of vitreous carnation shoots are aquatic in nature (62, 122), have a higher water content and consist of spongy parenchyma cells (122), and 96-98% or 100% of the shoot tips grown on solid or liquid media respectively, lack surface waxes (101).

These types of abnormal shoots usually fail to survive or root

following transfer from culture (5, 62, 101, 122), although Aitken et al. (5) reported that translucent Pinus radiata shoots developed normal growth if left on the same medium for several months. Fordham et al. (33) were able to root vitreous Rhododendron shoots which subsequently developed normally. Leshem (62) concluded that the failure of vitreous shoots to survive was due to their disorganized internal structure and loss of normal stomata. Rapid desiccation following transfer from culture may also be responsible for poor survival rates (101).

Ziv et al. (122) concluded that water availability in vitro was the key factor responsible for vitrification. This conclusion is supported by several lines of evidence. A resumption of normal plant growth in long term cultures normally associated with dehydration of the media has been reported by several investigators (5, 62, 122). Ziv et al. (122) also observed that carnation apices cultured on agar slants developed normal, glaucous leaves while those on horizontal agar, where water accumulated were vitreous.

High relative humidity in vitro has also been shown to cause vitreous leaf and shoot development (122). This was postulated by others (5, 101), but not supported. In culture, the relative humidity often approaches 100% (111, 122). This condition stimulates rapid, succulent growth and shoot proliferation, and may lead to the development of vitreous shoots. Ziv et al. (122) were able to increase the percent of normal carnation plantlets by reducing the relative humidity in vitro, and these plants were more capable of surviving transfer from culture than vitreous plants.

Another factor influencing vitrification is the water potential of the culture media (27, 62). Debergh et al. (27) measured the water potentials of media supplemented with varying agar, sucrose or mannitol

concentrations and found that increasing these supplements resulted in more negative water potentials. They were able to show that agar was responsible for the matric component of the media water potential and sucrose or manitol were responsible for the osmotic component. Interestingly, increasing the agar conentration was the only way to reduce vitrification. Increasing the sucrose or mannitol concentration had no effect. This can be explained by the fact that sucrose and mannitol are capable of penetrating tissue while agar cannot (27). Movement of sucrose or mannitol into the explants changes the osmotic balance between the tissue and medium and causes water to move into the tissue.

Sucrose in culture is both a carbon source and osmoticum (16). It also plays an important role in creating the critical turgor pressure required for cell expansion and shoot formation. In this respect, mannitol can substitute for sucrose but agar cannot. Fortunately, agar behaves in a different manner. Various hypotheses have been presented to explain the way agar effects shoot proliferation and vitrification but the most plausible is that increasing the the agar concentration creates water stress (62) in the tissue and makes it more difficult to attain the critical turgor required for growth (16). Other explanations for the role of agar include: an effect on the diffusion rate of molecules (88), with particular emphasis on cytokinins (27); the presence of an inhibitor or toxic substance in the agar (82, 88) and the inhibitory effect of an excessively hard gel (76).

In an interesting study, Singha (88) reported that agar played an important role in controlling shoot proliferation in cultures of Malus 'Almey' and Pyrus 'Seckel', but in different ways. Shoot proliferation in the Malus cultures was the greatest at 0.3% agar and decreased at

higher concentrations. Cultures of Pyrus, however, responded poorly at 0 or 0.3% agar with increased shoot proliferation only at higher agar concentrations. Singha (88) was somewhat at a loss to explain these results but it seems likely that these species respond differently to the availability of water.

Although increasing the agar concentration reduces vitrification and improves shoot quality it also decreases shoot proliferation (16, 27). Debergh et al. (27) observed that increasing the agar concentration in cultures of Cynara scolymus, Globe Artichoke, to 1.1% eliminated vitrification and greatly reduced shoot proliferation. This is a cause of concern because it may increase the cost of producing plants in vitro. However, if preventing vitrification increases the number of usable shoots then the control measure must be accepted.

Returning intact plants to their natural environment is the main objective of stage 3. This includes: developing an effecient rooting protocol, determining procedures for acclimating plants to ambient conditions and evaluating plants produced in vitro.

Auxins are normally required to stimulate root development in vitro (79). The three auxins used most often are: indoleacetic acid (IAA), indolebutyric acid (IBA) and naphthaleneacetic acid (NAA). These are listed in order of increasing physiological activity (76).

Hasagawa (41) studied the effect of IAA, IBA and NAA on the rooting of Rosa hybrida shoots. He found that IAA and NAA at 1.0 mgl^{-1} and 0.03 or 0.1 mgl^{-1} respectively were more effective than IBA. In a separate study, Snir (96) observed that 100% of Prunus avium shoots rooted on NAA media following a 14 day culture period compared to 30% on IBA, however, if left for a longer period of time all shoots eventually rooted.

An undesirable side effect of NAA is that it often stimulates callus development (59, 87). Lane (59) reported that concentrations of NAA above 0.1 mgl^{-1} stimulated callus growth and reduced rooting of Prunus and Spirea microcuttings.

Lowering the salt concentrations in MS media to 1/4 or 1/2 stimulates rooting in some Rosaceous species (41, 54, 59). Hyndman et al. (46) concluded that this stimulatory effect was mainly due to the reduction in nitrogen concentraion of the media.

Light has been shown to have a negative effect on rooting of some Rosaceous species (38, 79). Norton and Boe (79) suggested that this was due to the accumulation of endogenous IAA in the dark.

Deleting agar from the rooting medium has been shown to stimulate rooting of Grevillea (10), Prunus and Spirea (59) and Vitis (39) shoots. No reasons were given by these authors for the inhibitory effect of agar, but it may be due to its effect on the water potential of the media or to the presence of toxic substances in the agar (82).

Other procedures which stimulate root formation in vitro include: dipping Malus shoots in 0.1 mgl^{-1} IBA and culturing them in an inverted position (3) and supplementing the rooting media with 162 mgl^{-1} phloroglucinol (48, 49).

With some species (33, 35, 63, 65, 66, 69), it has been possible to root microcuttings under septic conditions. To prevent desiccation the microcuttings must be placed in a high humidity chamber or under mist.

It is interesting, that in most of these instances no hormone treatments were required to stimulate rooting. This was true even in species which are normally difficult to root (69). McCown and Amos (69) suggested that this was due to a change in the physiological state of the micropropagated shoots. Lyrene (66) concluded that the ease of

rooting was due to the phenomenon of rejuvenation.

Evaluation of woody plant populations propagated *in vitro* has been limited, partly due to the small numbers of plants propagated. Other problems which have undoubtedly limited evaluations are: space limitations, the extended period of time to grow plants to maturity and the use of seedling explants, where some segregation would naturally be expected.

Zimmerman (121) suggested that tissue cultured plants should be evaluated by their phenotypic appearance and field performance. Swartz et al. (102) successfully used these criteria to evaluate clones of *Fragaria* x *ananassa* cultivars. With few exceptions, they observed that the phenotypes of the clones were the same as plants propagated by standard techniques. The tissue culture clones did, however, exhibit some increased vigor and axillary bud activity. These differences may be due to epigenetic or phase changes (102, 121). Krul and Myerson's (56) observations with *Vitis* 'Seyval' support this argument. They observed that vines regenerated from callus by somatic embryogenesis were phenotypically identical but more closely resembled the original description of the cultivar than the parent plants.

Summary

The phenotype of a plant is controlled by its genotype and growth environment. This applies to explants in culture just as it does to plants in nature. The *in vitro* culture environment, however, is more closely controlled and can be easily manipulated by changing various factors including plant growth regulators.

Establishing aseptic, actively growing cultures can be a difficult

task depending upon plant species, condition of the plant material and time of year. Two problems which must be overcome are: surface contamination and phenolic secretion.

Stimulating rapid shoot proliferation in vitro is genotype and growth regulator dependent. Some genotypes respond more readily than others, with differences sometimes apparent even between closely related cultivars. Some species have been shown to become more responsive with increasing time in culture. This may be due to the phenomenom of rejuvenation. Plants recovered from these cultures often exhibit juvenile morphological characters.

Media development is an empherical process requiring a screening of components. In the future, it may be possible to select the appropriate media by analyzing endogenous hormone levels. This technology, however, still remains to be developed.

As noted earlier, BA is the most stimulatory cytokinin for promoting shoot proliferation in cultures of woody species.

Auxins are generally required to induce root development in vitro, although this is not always the case. With some species this is a simple task, however, for the majority this requires some effort.

Two areas which require additional emphasis are: developing procedures for acclimating and evaluating plants on a commercial scale.

Micropropagation of woody species is now in its infancy but with research concentrated on the areas previoulsy mentioned, should grow dramatically in the near future.

MATERIALS AND METHODS

Culture Initiation

Actively growing shoot tips, 7-10 cm, were removed during the spring growth period from flowering 6-8 year-old trees of Acer rubrum 'Red Sunset', growing at Beaumont Nursery, Michigan State University, East Lansing, Michigan. The shoot tips were cut into single node segments and shoot tips, 3-4 cm, the expanded leaves were removed and the explants were immersed in cold running tap water for 5 minutes. The explants were surface sterilized by immersion in a 10% Clorox solution plus 0.05% Tween 20 for 30 minutes followed by 5 rinses with sterile distilled water. The last rinse was decanted and the explants were held in a covered glass bowl until being prepared for culture.

The shoot tips and node segments, 3-4 cm, were trimmed at the base and placed on a slant in 100 x 15 mm Petri® dishes, 5 shoot tips or node segments/dish. The Parafilm® sealed dishes received a photon flux fluence rate of 34 $\mu E\ m^{-2}s^{-1}$ (G.E. F96T12CW, 400-700nm) during a 16 hr photoperiod at 25 $\pm$ 2° C. The culture medium was a modified Linsmaier and Skoog (LS) (3) with thiamine HCl 1.0, myo inositol 100, sucrose 30,000, agar (Sigma grade IV) 8000 mgl^{-1} and pH 5.8. The medium was autoclaved at 15 lb in^{-2} (1035 kPa) for 20 minutes, cooled to 45° C and dispensed into 100 x 15 mm Petri® dishes, 25 ml/dish.

To control phenolic secretion, shoot tips 3-4 cm, were: a) soaked in filter sterilized (0.45 μm Millipore filter) citric acid, 0, 150 or

300 mgl^{-1} for 30 minutes following surface sterilization and then held in a humid environment until being placed in culture or b) transferred to fresh LS medium with 0 or 1.0 [mgl^{-1}] 6(ɣ-ɣ-dimethylallylamino)-purine (2ip), 6-benzylaminopurine (BA) or 6-furfurylaminopurine (Kin) at 3 day intervals. Partially damaged explants were retrimmed to remove the injured tissue and returned to culture and dead or contaminated explants were discarded. After 3 or 4 transfers, actively growing shoots which had stopped secreting phenols were transferred to shoot proliferation medium.

Shoot Proliferation

In a preliminary experiment, axillary shoot proliferation was observed on *A. rubrum* 'Red Sunset' shoot tips after a 6 week culture period on LS + 10 mgl^{-1} 2ip. Proliferating stock cultures were established by culturing actively growing shoot tips on this medium for one or more culture periods. Shoot tips, 1.5 cm, from these cultures were used to initiate shoot proliferation and vitrification experiments. Except where noted, shoot tips were cultured singly for 4 wks in 60 ml bottles, then transferred intact to 240 ml bottles for 2 wks.

To determine the effect of cytokinins on shoot proliferation, shoot tips were placed on LS + 0, 1.0, 5.0, 10.0, or 15 mgl^{-1} 2ip, BA or Kin. Shoot tips were also placed on LS + 10 mgl^{-1} 2ip supplemented with 0, 50, 100, or 200 mgl^{-1} adenine sulfate or casein hydrolysate.

In a separate set of experiments randomly selected shoot tips, 1.5 cm, were grown for 4 wks in 60 ml bottles on LS + 10.0 mgl^{-1} 2ip, decapitated 2 nodes above the basal callus mass and returned to 60 ml bottles. Shoots were harvested from the decapitated cultures at 10 day

intervals.

Shoot tips, 1.5 cm, were placed on LS + 10.0 mgl^{-1} 2ip, with the following modifications or treatments to determine their effect on shoot proliferation and vitrification: a) sucrose 10, 20, 30, 40, or 50 gl^{-1}, b) agar 4, 6, 8, 10, or 12 gl^{-1}, c) salt concentration 25, 50, 75, or 100%, d) temperature 20° C for 0, 1, 2, 3, or 4 wks followed by 25° C for a total of 6 wks, e) comparison of 25 x 150 mm culture tubes cultured on a slant with 60 ml bottles, 4 wk culture period and f) comparison of 60 and 240 ml bottles; shoot tips were grown for 4 wks, decapitated 2 nodes above the basal callus and returned to the same size culture vessel for 2 wks.

Three culture vessels, 60 and 240 ml glass bottles with metal screw caps and 25 x 150 ml glass culture tubes capped with plastic Kaputs® were used in the experiments with 25, 50 and 25 ml media/vessel respectively. The media were heated to dissolve the agar, dispensed into the culture vessels and autoclaved as described previously. One shoot tip was placed in each culture vessel with 5 or 10 replications/treatment. The experiments were arranged in a completely randomized design and were repeated at least twice. Data were analyzed using the F test with means separated using Duncan's Multiple Range Test or Student's T test ($P \leq 5\%$). Results from duplicate experiments were combined unless there were significant differences, then the data were presented separately.

Rooting, Acclimation and Evaluation

To determine the effect of auxins on root development, shoot tips, 1.5 cm, from aseptic stock cultures were placed on LS + 0, 0.01, 0.1 or

1.0 mgl^{-1} indolebutyric acid (IBA), naphthaleneacetic acid (NAA) or indoleacetic acid (IAA) for 2 wks. Each treatment was replicated 10 times and the experiments were repeated at least twice. Experiments were designed and the data analyzed as described previously.

One shoot tip was placed in each 25 x 150 mm glass culture tube with 25 ml media/culture tube. All components of the media were autoclaved except for IAA which was filter sterilized (0.45μm Millipore filter) and added to cooled (45° C) media. Shoot tips were also rooted in 0.95 l bottles, 50 shoot tips/bottle. Each bottle contained 75 ml LS basal medium and the aluminum foil sealed bottles were placed in a horizontal position.

All cultures recieved a photon flux fluence rate of 34μE $m^{-2}s^{-1}$ (G.E. F96T12CW, 400-700 nm) under a 16 hr photoperiod at 25 $\pm$ 2 C.

Rooted shoots were removed from culture, washed in tap water to remove the agar and planted in AC-6/12 Cell Paks® (Ball Seed Co., West Chicago, Illinois) using a peat-lite planting mix (VSP, Bay Houston Towing Co.). The Cell Paks® were placed inside plastic bags to prevent dessication and were held in a growth room at a photon flux fluence rate of 38-64μE m^{-2} s^{-1} (G.E. F96T12CW, 400-700nm) under a 16 hr photoperiod at 25$\pm$2 C. The plants were acclimated to the ambient environment by gradually opening the plastic bags over a 2 wk period and were then transferred to the greenhouse.

Plants in the greenhouse were grown under a natural photoperiod supplemented with a 4 hr night break, 10:00 p.m.-2:00 a.m. of 21.5μE m^{-2} s^{-1} (G.E. 100w incandescent) from September 15-March 15. The day and night temperature was 23° C minimum and the day temperature fluctuated higher with the season. Plants were fertilized once/week with 150 ppm N, 20-20-20 using acidfied (H_3PO_4) water to maintain the

planting medium at pH 6.5. After 2 wks, the plants were transferred to 3.8 l plastic pots using the same planting mix. Plant height was measured biweekly and the plants were evaluated visually.

RESULTS

The establishment of aseptic, actively growing cultures of A. rubrum 'Red Sunset' was hindered by surface contamination and the secretion of polyophenols. Explants collected in the spring during the first growth flush were readily surface sterilized; whereas, those collected later in the year were increasingly more difficult. Surface contaminants could be eliminated but the concentration of sterilant required also seriously damaged or killed the shoot tip or node segment explants. Injured explants secreted phenols from the wounded surfaces eventually resulting in death of the affected tissue (Fig. 1). This was accompanied by browning of the surrounding medium.

The only effective method found for controlling phenolic secretion was the use of a preconditioning treatment. This treatment consisted of subculturing undamaged or partially damaged, 3-4 cm shoot tips or node segments to fresh LS basal medium at 3-4 day intervals. Damaged tissues and dead or contaminated explants were discarded. Addition of 1.0 mgl^{-1} 2ip or BA to the preconditioning medium stimulated basal callus and new shoot growth. In this respect BA was more effective than 2ip. Kinetin at this concentration was not effective in promoting callus or shoot growth. After 3-4 subcultures the phenolic secretion would cease and the explants could be transferred to Stage 2 experimental media.

Attempts to control phenolic secretions were also made by soaking explants in 150 or 300 mgl^{-1} citric acid for 30 minutes following surface sterilization. However, this treatment resulted in further

Figure 1. Phenolic secertion from Acer rubrum 'Red Sunset' node segments.

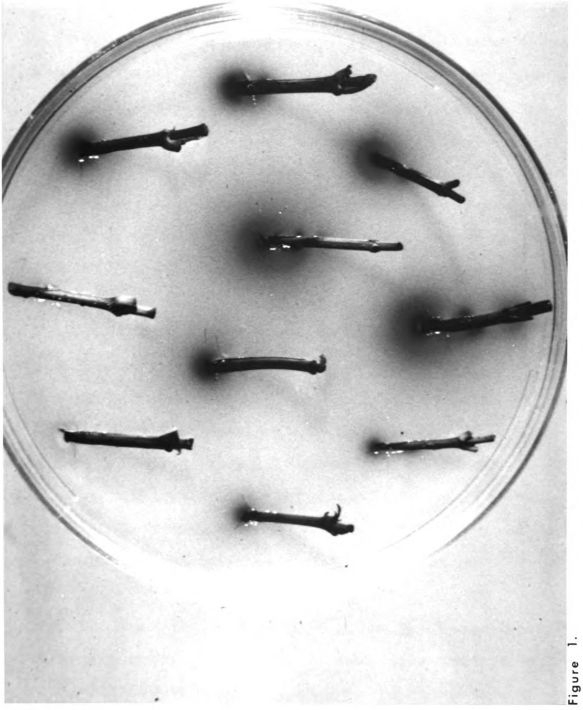

Figure 1.

injury. Improved explant survival could be obtained by holding the shoot tips or node segments in a covered glass bowl before dissection rather than floating in the last water rinse.

Shoot Proliferation

The morphogenetic responses of *A. rubrum* 'Red Sunset' shoot tips to 2ip, BA and Kinetin are shown in Table 2. BA was the most consistent of the three cytokinins tested in stimulating shoot proliferation with 5.9 shoots/culture at 5.0 mgl^{-1}, although 2ip was nearly as effective with 3.0 shoots/culture at 10.0 mgl^{-1}. With both of these cytokinins, axillary bud break was observed during the third or fourth week of culture (Fig. 2A) followed by shoot elongation during the next two week culture period (Fig. 2B). Axillary bud break was also observed at the lowest node of shoot tips cultured on 10.0 mgl^{-1} Kinetin, but subsequent shoot elongation did not occur. Adventitious shoot development was not observed with any cytokinin treatment.

The requirement for a high level of cytokinin to stimulate axillary shoot development in 'Red Sunset' cultures can be seen in Fig. 3. Shoots placed on LS medium lacking cytokinins rapidly developed roots and grew slowly with little or no shoot elongation during a 4 week culture period. In comparison, shoot tips placed on LS + 10.0 mgl^{-1} 2ip elongated and axillary bud break occurred during the same period of time.

Mean shoot length (Table 2) varied significantly depending on cytokinin concentration. With 2ip and BA, shoot length was the greatest at the concentration just below the optimum for shoot proliferation.

2ip and BA stimulated large amounts of callus a the base of the

Table 2. The morphogenetic responses of Acer rubrum 'Red Sunset' shoot tips after 6 wks culture in LS medium containing the cytokinins, 2ip, BA and kinetin.

Cytokinin (mgl^{-1})	Shoot Proliferation %	Mean Shoot Number[x]	Damaged Shoots %	Mean Shoot Length (cm)[x]	Mean Callus Fresh Wt. (gm)[x]
2ip					
0	0	1.0 b	0		
1.0	0	1.0 b	0	1.9 b	0.53 b
5.0	20	1.3 b	0	6.1 a	3.02 a
10.0	50	3.0 a	33	3.3 b	3.21 a
15.0	10	1.1 b	9	1.9 b	0.71 b
BA					
0	0	1.0 b	0		
1.0	70	2.4 b	8	4.5 a	0.81 b
5.0	80	5.9 a	25	2.6 b	3.01 a
10.0	30	1.4 b	7	1.5 bc	1.31 b
15.0	10	1.1 b	0	1.3 c	0.55 b
Kin					
0	0	1.0 a	0		
1.0	0	1.0 a	0	1.6 b	0.10 b
5.0	10	1.1 a	0	2.4 b	0.43 a
10.0	0	1.0 a	0	1.9 ab	0.42 a
15.0	0	0.8 a	0	1.1 c	0.36 a

[x]Means for a given cytokinin and column with the same letter are not significantly different. Duncans Multiple range ($P \leq 5\%$).

Figure 2. Acer rubrum 'Red Sunset' shoot tips. A. Axillary bud break after 4 wks on LS + 10.0 mgl^{-1} 2ip. B. Axillary shoot elongation after 6 wks on LS + 10.0 mgl^{-1} 2ip.

Figure 2.

Figure 3. Growth of *Acer rubrum* 'Red Sunset" shoot tips after 4 wks on LS + 10.0 mgl^{-1} 2ip (left) or LS basal medium (right).

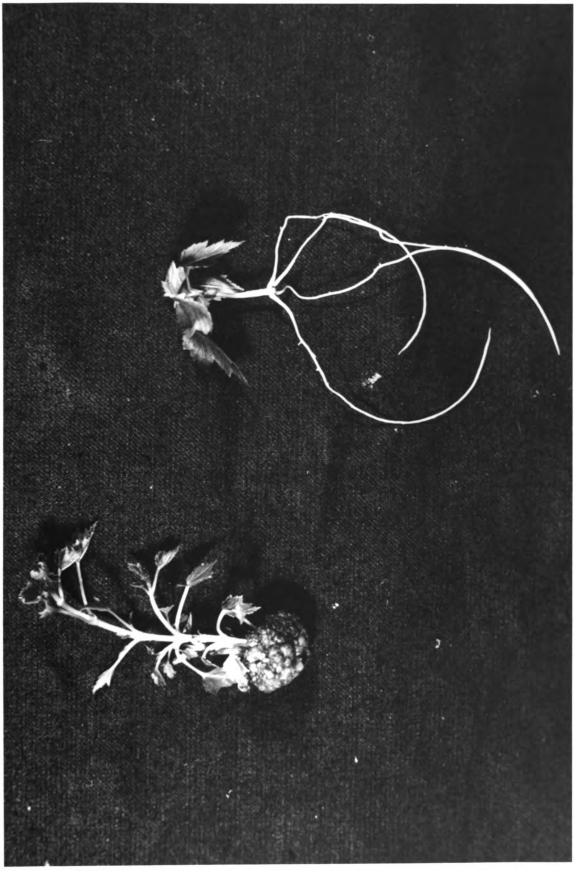

Figure 3.

shoot tip explants. Callus fresh weight varied significantly depending on the cytokinin concentration. The callus was compact, nodular and whitish- or reddish-green.

Vitreous shoots were observed in several of the 2ip and BA treatments, with substantial damage occurring at the concentrations optimum for shoot proliferation. Symptoms of vitrification included: translucent leaves and shoot tips; succulent, water soaked tissues and guttation from leaves and shoot tips; followed by death of the shoot tips and tissue collapse. In some cases, stem swelling, compact internodes and intumescences from lenticels were also observed. Shoots with vitreous symptoms did not survive transfer to fresh medium. Damage normally occurred first at the apex of the primary shoot and proceeded toward the base. Death of the shoot terminals often stimulated shoot proliferation. However, if left unattended, entire cultures would succumb to these symptoms. In some instances, normal growth would resume if cultures were held for eight to ten weeks without subculturing.

Decapitating A. rubrum 'Red Sunset' shoot tips stimulated axillary shoot proliferation (Table 3 and Fig. 4). Shoot proliferation was more consistent using this procedure than when shoot tips were left intact. The terminal shoot tips could be returned to fresh medium to reinitiate the shoot proliferation cycle. Shoots, 1.0 cm or larger could be harvested 10 days later. The number of vitreous shoots, however, increased at the second harvest date. Harvesting shoots at intervals greater than 10 days also increased the number of damaged shoots. These shoots were succulent and tender and easily damaged by adverse conditions.

Fasciated shoots and shoots with 3 leaves/node were occasionally

Table 3. The morphogenetic responses of Acer rubrum 'Red Sunset' shoot tips to decapitation. The shoot tips were cultured for 4 wks on LS + 10.0 mgl^{-1} 2ip, decapitated and basal sections were returned to the same medium with shoots harvested at 10 day intervals.

Number Cultures	Shoot Proliferation (%)	Mean Shoot Number Harvest 1	Mean Shoot Number Harvest 2	Damaged Shoots (%)	
	30	97	3.8		1
15	100	5.7		0	
11	100	5.7	5.3	6	17
15	100	5.7	6.5	0	0

Figure 4. Acer rubrum 'Red Sunset' shoot tip explants 2 wks after decapitation, 4.0 shoots/explant. The shoot tips were cultured for 4 wks on LS + 10.0 mgl^{-1} 2ip, decapitated and returned to the same medium for 2 wks.

Figure 4.

observed in the decapitated cultures. These abnormal shoots were easily identified and removed.

The morphogenetic responses of 'Red Sunset' shoot tips to media amended with adenine sulfate or casein hydrosylate are shown in Table 4. The mean shoot number was significantly increased by the addition of 100 mgl^{-1} adenine sulfate to the culture medium compared to the control, LS + 10.0 mgl^{-1} 2ip, while 200 mgl^{-1} had a toxic effect. Mean callus fresh wt. decreased significantly at adenine sulfate concentrations above 50 mgl^{-1}. Significant experiment by treatment interactions in mean callus fresh weight were observed between the three adenine sulfate experiments (Fig. 5). Casein hydrosylate, at the levels tested, had no effect on the growth of A. rubrum 'Red Sunset' shoot tips.

Shoot Proliferation and Vitrification

Acer rubrum 'Red Sunset' shoot tips responded differentially to sucrose or agar concentrations (Table 5). The percent shoot proliferation and mean shoot number increased with increasing sucrose concentration although there was no significant difference between 30, 40 or 50 gl^{-1}. Culturing shoots on 10 gl^{-1} sucrose severely restricted shoot proliferation, shoot elongation and callus growth. Increasing the sucrose concentration above 30 gl^{-1} did not reduce the incidence of vitreous shoots. Callus fresh weight increased significantly with increasing sucrose concentration.

As the sucrose concentration of the LS + 10.0 mgl^{-1} 2ip medium was increased the amount of anthocyanin in the shoot tip explants increased

Table 4. The morphogenetic responses of Acer rubrum 'Red Sunset' shoot tips after a six week culture period in LS + 10.0 mgl^{-1} 2ip and adenine sulfate or casein hydrolysate.

Supplement (mgl^{-1})	Shoot Proliferation (%)	Mean Shoot Number[x]	Damaged Shoots (%)	Mean Shoot Length (cm)[x]	Mean Callus Fresh Wt. (gm)[x]
Adenine sulfate					
0	53	2.1 bc	6	4.59 a	4.02 a
50	53	3.7 ab	11	4.48 a	3.91 a
100	67	4.4 a	14	3.81a	2.73 b
200	40	1.4 c	0	3.48 a	0.97 c
Casein hydrosylate					
0	47	2.8 a	14	3.84 a	4.24 a
50	47	2.9 a	20	3.67 a	3.83 a
100	40	2.3 a	17	3.21 a	3.51 a
200	40	2.4 a	22	2.96 a	3.32 a

[x]Means for a given supplement and column with the same letter are not significanlty different. Duncans Multiple Range ($P \leq 5\%$).

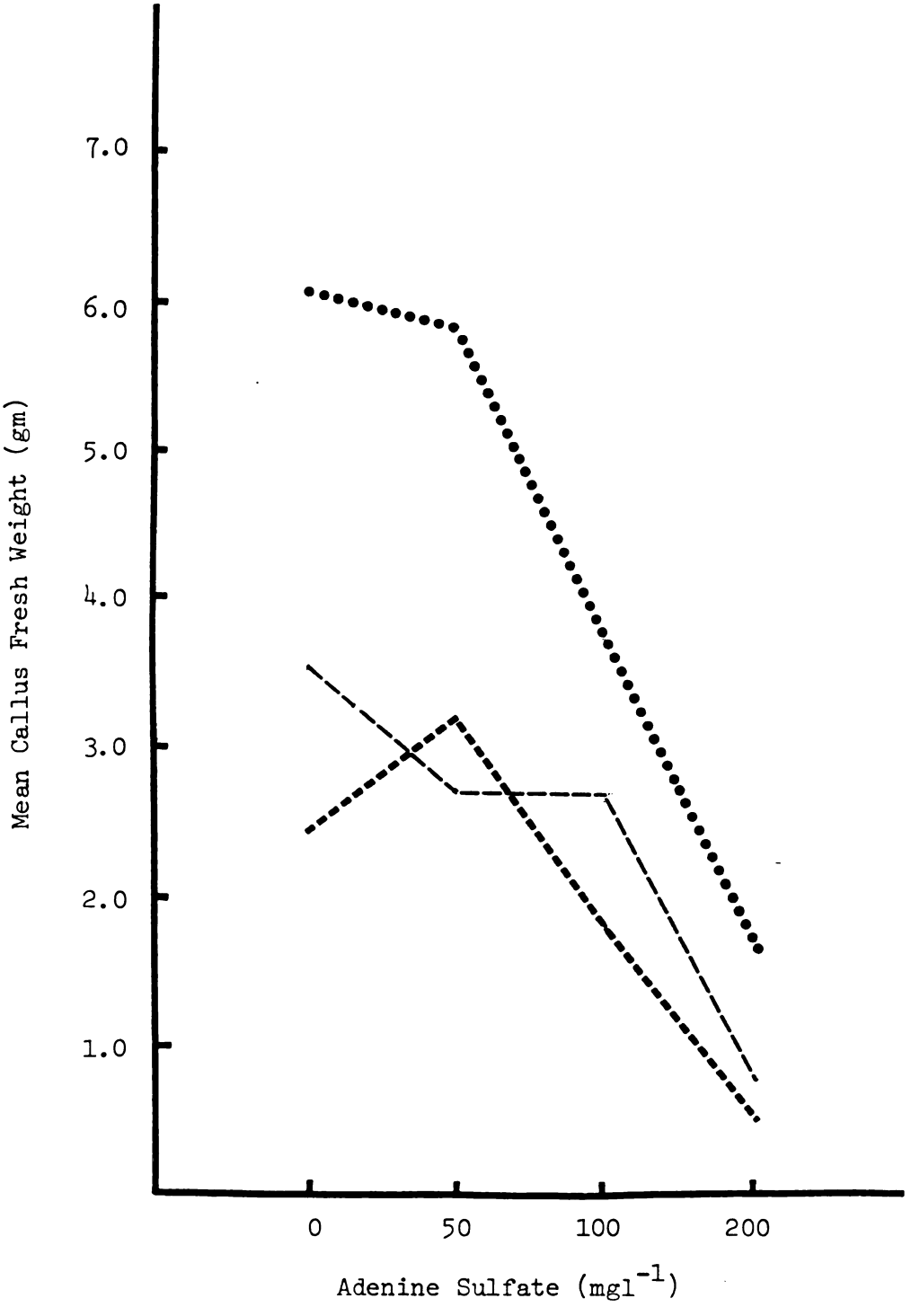

Figure 5. The effect of adenine sulfate on basal callus formation in Acer rubrum 'Red Sunset' shoot tip cultures after 6 wks on LS + 10.0 mgl^{-1} 2ip. Significant experiment by adenine sulfate concentration interactions for mean callus fresh weight were observed between the three experiments: 1——, 2•••and 3▪▪▪.

Table 5. The morphogenetic responses of Acer rubrum 'Red Sunset' shoot tips after a six wk culture period in LS + 10.0 mgl^{-1} 2ip and varying sucrose or agar concentrations.

Treatment (gl^{-1})	Shoot Proliferation (%)	Mean Shoot Number[x]	Damaged Shoots (%)	Mean Shoot Length (cm)[x]	Mean Callus Fresh Wt. (gm)[x]
Sucrose					
10	0	1.0 c	0	1.70 c	0.17 d
20	20	1.4 bc	0	4.89 a	1.23 c
30[y]	60	3.7 ab	16	3.18 b	3.30 b
40	80	3.5 ab	11	4.81 a	4.43 a
50	100	4.2 a	33	3.84 ab	4.34 a
Agar					
4	30	2.3 b	22	4.43 a	2.29 b
6	30	1.8 b	6	3.49 ab	2.10 b
8[y]	60	4.8 a	19	3.03 bc	2.74 ab
10	30	1.4 b	7	2.37 bc	3.16 a
12	20	1.3 b	0	1.99 c	2.90 ab

[x]Means for a given treatment and column with the same letter are not significantly different. Duncans Multiple range ($P \leq 5\%$).
[y]Control treatment.

and was highly visible at 50 gl^{-1}. Stems, petioles and leaves were reddish-green and some of the new shoots, especially those from lower nodes, were entirely red.

Shoot proliferation was most consistent at 8 gl^{-1} agar and the mean shoot number was significantly lower at agar concentrations below or above 8 gl^{-1}. Vitreous shoots were eliminated by raising the agar concentration to 12 gl^{-1}. Mean shoot length decreased significantly with increasing agar concentration.

A highly significant experiment by treatment interaction in callus fresh weight was observed between the two agar experiments (Fig. 6). Callus fresh weight was essentially the same at 4 or 6 gl^{-1} and varied significantly at higher concentrations.

Lowering the salt concentrations of LS + 10.0 mgl^{-1} 2ip medium by 25% significantly reduced shoot proliferation and mean shoot number (Table 6). Shoot length and callus fresh weight were also decreased by lowering the salt concentration but the reduction as gradual. Decreasing the salt level had the least effect on callus fresh weight.

Holding A. rubrum 'Red Sunset' shoot tips at 20° C for at least two weeks before transfer to 25° C significantly reduced shoot proliferation, mean shoot number and callus fresh weight. Increasing the length of the 20° C treatment to 3 or 4 weeks had a deleterious effect on all parameters.

A significant experiment by temperature interaction was observed in mean shoot number between the two experiments (Fig. 7).

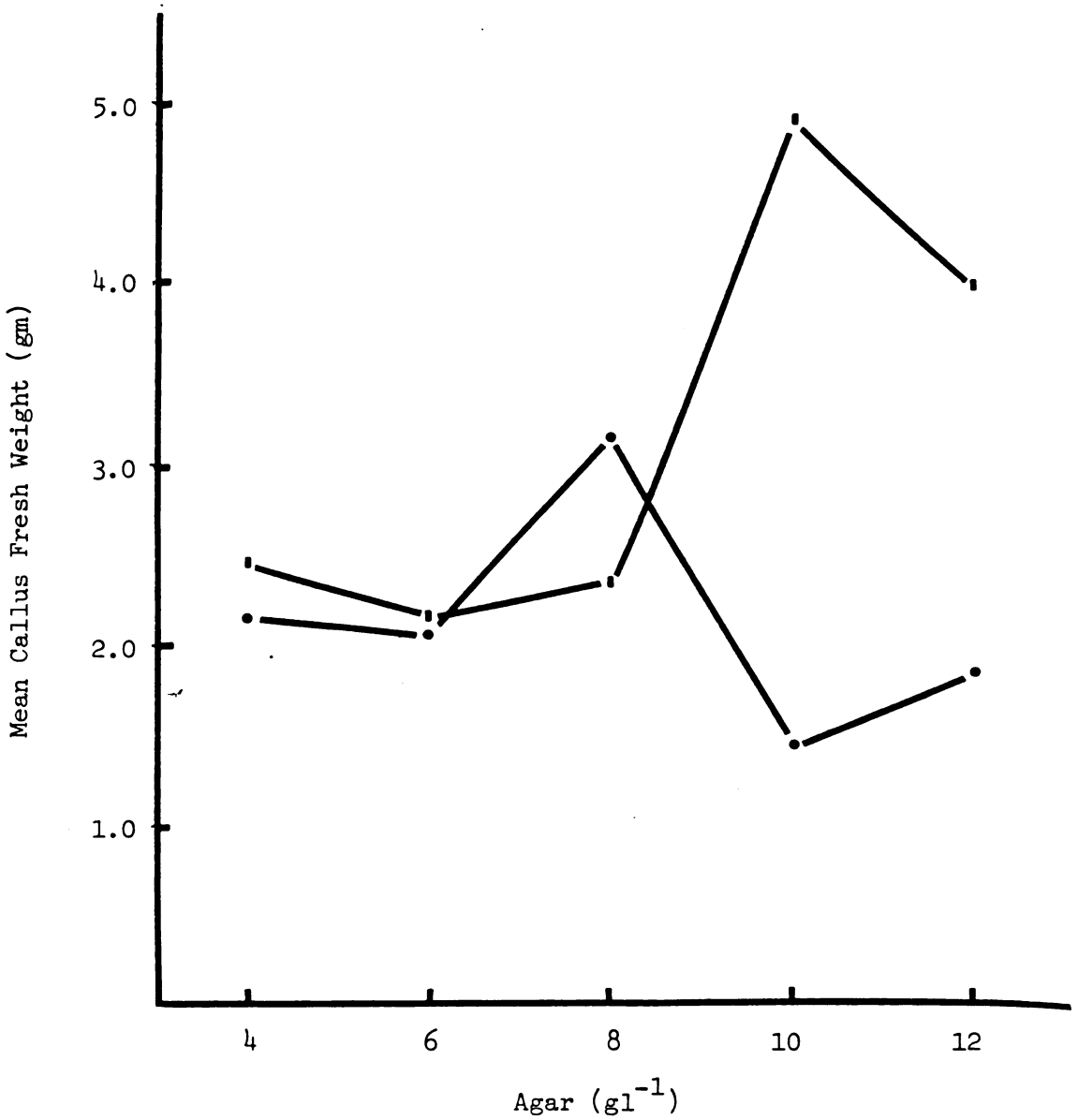

Figure 6. The effect of varying agar concentrations on basal callus formation in Acer rubrum 'Red Sunset' shoot tip cultures after 6 wks on LS + 10.0 mgl^{-1} 2ip. A highly significant experiment by agar concentration interaction for mean callus fresh weight was observed between experiment: 1 (•) and 2 (▮).

Table 6. The morphogenetic responses of *Acer rubrum* 'Red Sunset' shoot tips to variable salt concentrations or temperature regimes. Basal medium was LS + 10.0 mgl^{-1} 2ip. Shoot tips in the temperature experiment were held for 0 - 4 wks at 20° C then transferred to 25° C. Data were taken after a 6 wk culture period.

Treatment	Shoot Proliferation (%)	Mean Shoot Number	Damaged Shoots (%)	Mean Shoot Length (cm)[x]	Mean Callus Fresh Wt. (gm)[x]
Salt Concn. (%)					
100[y]	80	4.7 a	4	4.92 a	3.44 a
75	0	1.0 b	0	3.93 a	3.24 a
50	20	1.1 b	0	1.93 b	2.40 a
25	10	0.9 b	11	1.14 b	0.50 b
Temperature 20° C wks					
0[y]	80	5.0 a	26	4.17 a	3.25 a
1	70	3.9 ab	26	4.20 a	3.04 a
2	50	2.3 bc	43	3.31 ab	1.84 b
3	20	1.9 bc	11	2.14 b	1.32 bc
4	0	1.0 c	0	2.23 b	0.93 c

[x]Means for a given treatment and column with the same letter are not significantly different. Duncans Multiple range ($P \leq 5\%$).
[y]Control treatment.

Mean shoot number and shoot length in 25 x 150 mm culture tubes and 60 ml bottles were not significantly different as analyzed by Student's t test ($P \leq 5\%$) (Table 7). However, explant quality was better in 60 ml bottles than in the culture tubes. Leaves and shoots in 60 ml bottles were green and appeared normal while many of those in the culture tubes had a waterlogged, translucent appearance. Shoot tips in the culture tubes had a tendency to grow upwards against the glass where water condensed. This situation often led to death and collapse of the shoot tips. Death of the shoot terminals enhanced axillary bud break and shoot elongation. The gain in shoot proliferation, however, was negated by the spread of vitreous symptoms to the new growth.

After a four week culture period in 60 or 240 ml bottles, there was a marked difference in the growth of 'Red Sunset' shoot tips. Axillary shoot elongation was evident in 50% of the cultures in 60 ml bottles compared to 10% in 240 ml bottles. New shoot growth was evident in both culture vessels but internodes and petioles were more compact in 240 ml bottles. Explants growing in 240 ml bottles had a hardened appearance.

The same trends in shoot proliferation and elongation were evident following decapitation (Table 7). Mean shoot length was significantly reduced in 240 ml bottles compared to 60 ml bottles. The number of damaged shoots after the six week culture period was lower in 60 ml than 240 ml bottles. Mean callus fresh weight was significantly greater in the 240 ml bottles than in the 60 ml bottles.

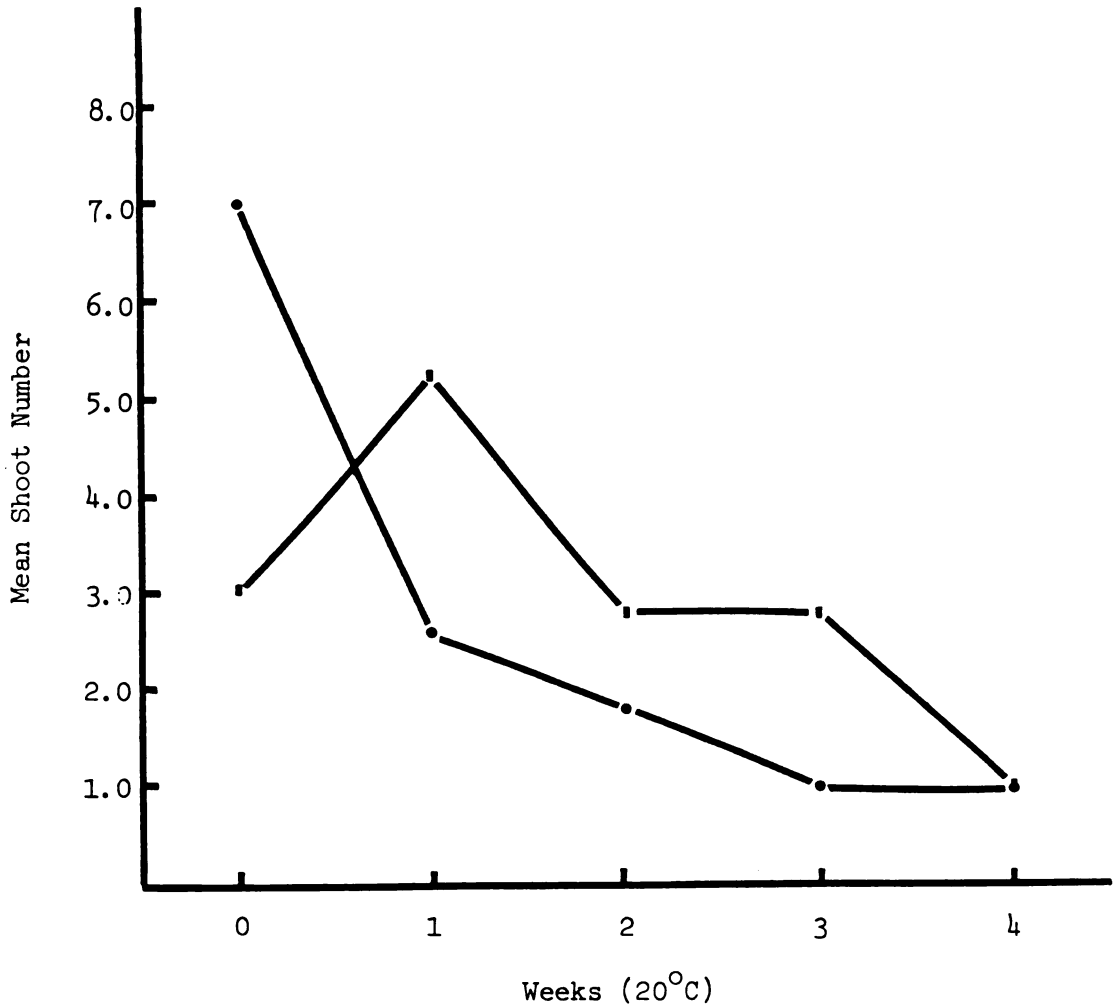

Figure 7. The effect of varying temperature regimes on axillary shoot proliferation in cultures of Acer rubrum 'Red Sunset'. Shoot tip explants were held for 0-4 wks at 20°C followed by transfer to 25°C for a total of 6 wks. Basal medium was LS + 10.0 mgl^{-1} 2ip. A significant experiment by temperature interaction in mean shoot number was observed between experiment: 1 (•) and 2 (▪).

Table 7. The morphogenetic responses of Acer rubrum 'Red Sunset' shoot tips in different culture vessels. Shoot tips were cultured in LS + 10.0 mgl^{-1} 2ip. In the six wk experiment, shoot tips were cultured intact for four wks, then decapitated two nodes above the base and the basal mass was returned to the same culture vessel for two weeks.

Culture Vessel	Shoot Proliferation (%)	Mean Shoot Number	Damaged Shoots (%)	Mean Shoot Length (cm)[x]	Mean Callus Fresh Wt. (gm)[x]
Four Weeks					
25 x 150 mm Culture Tubes	45	2.8	27	3.01	____
60 ml Bottles	30	1.8	0	3.46	____
Six Weeks					
60 ml Bottles	100	4.7	4	3.04**	3.34
240 ml Bottles	50	2.7	30	1.35	5.88**

[x]Students' t test ($P \leq 5\%$).

Rooting and Plant Evaluation

'Red Sunset' shoot tips rooted readily during a two week culture period on LS basal medium or LS supplemented with IBA, NAA or IAA (Table 8). Roots were visible in all treatments 5-6 days after the shoot tips were placed in culture. The roots generally arose from the base of the shoot tips without intervening callus. The adventitious roots were white and appeared normal (Figure 8), although there were no secondary roots or root hairs. Raising the concentrations of IBA or NAA to 1.0 mgl^{-1}, significantly increased the mean root number and decreased root length. At this concentration, these auxins stimulated callus formation which was quickly followed by root development. Roots were observed at the base of the shoot tips on 1.0 mgl^{-1} NAA after 3 days culture. The roots were short, abnormally thickened and brittle.

Little or no new shoot growth was observed following transfer of shoots to rooting media. The internodes were compact and the leaves were green or dark-green.

'Red Sunset' shoots also rooted with 100% efficiency in 0.95 liter bottles.

Acer rubrum 'Red Sunset' plants growing in the green house were identical (Fig. 9) with the exception of occasional plants with 3 leaves/node. This was true even of plants from cultures 2.5 years-old. The plants grew rapidly following transfer from culture (Fig. 10). The leaves were 5 lobed and heart shaped at the base. This is in contrast to leaves of parent plants which were generally 3 lobed and rounded at the base. After 18 weeks the plants had reached a height of 82.9 cm. Rapid growth was also observed in containers under field conditions with adequate nutrition.

Table 8. The effect of the auxins, IBA, NAA or IAA incorporated in LS medium on rooting of Acer rubrum 'Red Sunset' shoot tips after a 2 wk culture period.

Auxin (mgl^{-1})	Rooting (%)	Mean Root Number[x]	Mean Root Length (cm)[x]
IBA			
0	85	2.4 b	2.30 a
0.01	100	2.7 b	2.37 a
0.1	100	3.3 b	2.01 a
1.0	95	10.4 a	0.72 b
NAA			
0	100	2.7 b	2.78 a
0.01	100	2.6 b	2.26 a
0.1	100	3.6 b	1.81 b
1.0	95	6.4 a	0.43 c
IAA			
0	100	3.0 a	2.15 a
0.01	100	4.0 a	1.90 ab
0.1	100	3.3 a	2.37 a
1.0	100	4.0 a	1.47 b

[x]Means for a given auxin and column with the same letter are not significantly different. Duncans Multiple Range ($P \leq 5\%$).

Figure 8. Rooted Acer rubrum 'Red Sunset' shoot tips 2 wks after being placed on LS basal medium.

Figure 8.

Figure 9. *Acer rubrum* 'Red Sunset' plants propagated *in vitro*; 4 wks after transfer from cultures to planting medium, height 4.1 cm.

Figure 9.

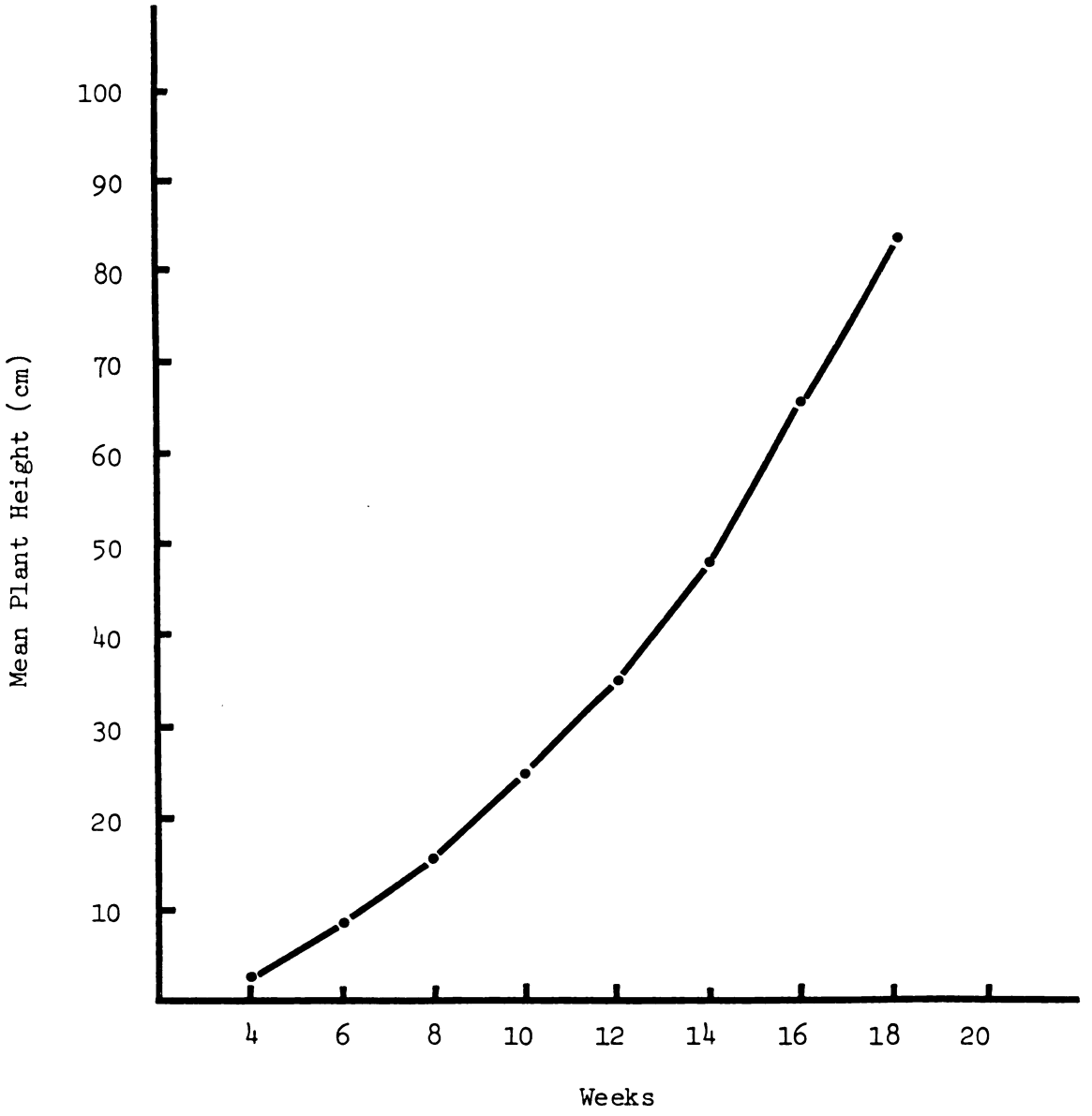

Figure 10. Growth rate of *in vitro* propagated *Acer rubrum* 'Red Sunset' plants in the greenhouse. Four weeks after transfer from culture, uniform plants were transplanted in 3.8 liter plastic pots using a peatlite planting mix (VSP, Bay Houston Towing Co.). The plants were fertilized once/week with 150 ppm N, 20-20-20 during the growing period from 4/13/82-8/23/82.

Plantlets transferred to the greenhouse during November, December and January grew poorly and often became dormant. The growth rate of older plants also slowed during this period of time and most of the plants became dormant.

DISCUSSION

The most difficult stage in propagating Acer rubrum 'Red Sunset' in vitro was the initiation of aseptic, actively growing cultures. As the name of this cultivar implies, a large amount of anthocyanin is produced in the tissues. This red coloration is particularly evident on new stem growth, flowers and fruit during the spring and on leaves during the fall. Surface sterilization of actively growing 'Red Sunset' shoot tips or node segments with a diluted Clorox solution injured the tissues and stimulated phenolic secretions. This is suggested because after several subcultures phenolic secretion ceased and did not reappear even upon dissection. Damaged explants rapidly turned brown and died if left unattended. The secretion of phenols from 'Red Sunset' explants was not entirely suprising, however, since anthocyanins and phenols share a common biosynthetic pathway (95). Similar difficulties were also encountered with attempts to establish cultures of A. ginnala in vitro (Meyer and Welsh, 1982 unpublished data). Shoot tips of this species collected during the spring growth flush are bright red and rapidly turning black following surface sterilization.

Phenolic secretion in 'Red Sunset' cultures could be controlled by using a preconditioning treatment (22). This treatment consisted of placing shoot tip or node segment explants, 3-4 cm, on LS + 10.0 mgl^{-1} BA or 2ip and transferring at 3-4 day intervals. These two cytokinins stimulated new shoot growth and also callusing of wound surfaces. Rapid subculturing allowed removal of damaged and dead tissue or contaminated

explants. Similar procedures have been used by others (14, 67) to effectively control phenolic secretion. Holding 'Red Sunset' explants in a humid environment prior to dissection also helped to reduce injury by polyphenols.

In some instances (7, 67), soaking explants in antioxidants or incorporating them in the culture medium has proven effective in controlling phenolic secretion. This was not observed, however, with A. rubrum 'Red Sunset'. Soaking 'Red Sunset' shoot tips or node segments in citric acid resulted in further injury. Broome and Zimmerman (14) made a similar observation with Rubus cultures. These responses may indicate that damage due to phenolic secretion was only secondary to damage caused by the surface sterilants. Damage control, therefore, should be aimed at modifying the surface sterilization procedure rather than at controlling end products.

High levels of BA or 2ip, 5.0 or 10.0 mgl^{-1} respectively, were required to overcome apical dominance in A. rubrum 'Red Sunset' cultures. At these concentrations, axillary shoot proliferation only occurred after the formation of a basal callus mass and elongation, of the shoot tip explants. Lower levels of BA, 1.0 mgl^{-1} or 2ip, 1.0 or 5.0 mgl^{-1} also stimulated basal callus formation and shoot elongation but shoot proliferation rates were significantly reduced. Both the high cytokinin requirement and the sequential growth pattern imply the presence of an endogenous hormone gradient in 'Red Sunset' shoot tips. Fari and Czako (32) made a similar observation with Capsicum annuum 'T. Hatvani' hypocotyl sections. They reported that shoot development was dependent on the position of the explants along the hypocotyl. Explants taken just below the cotyledons developed shoots while those closer to the root developed callus or roots. They concluded that differentiation

was controlled by the complement of exogenous and endogenous growth substances. This may also be the case with 'Red Sunset' shoot tip cultures. To stimulate axillary shoot development, the cytokinin levels in the medium had to be above a critical level. Since auxin(s) and cytokinin(s) interact in regulating the growth of axillary buds (61, 117), one possible explanation for these observations is that A. rubrum shoots produce a high level of endogenous auxin(s). This hypothesis is supported by several lines of indirect evidence: 1) young A. rubrum trees have a pyramidal growth habit (28) suggesting strong apical dominance, 2) shoot proliferation in vitro was most consistent when shoots on 10.0 mgl^{-1} 2ip were decapitated and 3) 'Red Sunset' shoots rooted readily in vitro on basal medium lacking auxins.

The most effective cytokinin in stimulating axillary shoot proliferation in 'Red Sunset' cultures was BA. This appears to be a general trend with many woody species (19, 40, 41, 64, 86, 90, 103). It was also possible to stimulate axillary shoot proliferation using 2ip but twice the optimum BA concentration was required. Haseqawa (41) made a similar observation with Rosa hybrida cultures where 2ip at 10.0 mgl^{-1} was nearly as effective as 1.0 mgl^{-1} BA in stimulating shoot proliferation.

One procedure commonly used to stimulate shoot proliferation in vitro is decapitation (14, 41, 54, 60, 86, 96, 100). This is due to the release of axillary buds from apical dominance (40, 96). In some instances, this procedure allows successive harvests of shoots from mother cultures (14, 60, 86, 100). This method was also effective with 'Red Sunset' cultures. Shoot proliferation was more consistent following decapitation than when shoots were left intact. It was possible to harvest shoots twice at 10 day intervals.

In an attempt to stimulate or improve shoot proliferation rates, various supplements including adenine sulfate (6, 26, 39, 41, 65) and casein hydrolysate (60, 65) are sometimes added to the culture medium. With 'Red Sunset' shoot tip cultures, addition of 100 mgl^{-1} adenine sulfate to the multiplication medium, LS + 10.0 mgl^{-1} 2ip, significantly increased axillary shoot proliferation and significantly reduced the amount of basal callus. These observations were interesting since 'Red Sunset' shoots on multiplication medium developed an excessive amount of callus at the base. These results suggested that this large mass of basal callus may have a negative impact on shoot proliferation.

Addition of casein hydrolysate to 'Red Sunset' multiplication medium did not stimulate shoot proliferation although Lyrene (65) reported that this compound improved shoot proliferation rates of Vaccinium ashei when used at 300 mgl^{-1}.

Maximum shoot proliferation was only observed in A. rubrum 'Red Sunset' shoot tip cultures when certain conditions were optimized. These essential factors were: high levels of BA or 2ip, high salt levels, adequate sucrose concentration, readily available free water, and proper temperature regime. Unfortunately, these favorable conditions also led to the development of vitreous shoots. The shoot terminals and leaves became translucent and water soaked, followed by tissue collapse and death. Guttation was normally associated with the onset of these conditions. This sequence was repeated in a cyclic pattern with the elongating axillary shoots which developed as the terminals senesced. In some instances, the entire shoot mass would succumb. Debergh et al. (27) observed similar symptoms in Cynara scolymus cultures and termed this phenomenon vitrification.

Anatomical studies of vitreous shoots and leaves indicate that they

are aquatic in nature (122) or are similar to plants growing under wet conditions (8, 62). Ziv et al. (122) noted that Dianthus caryophyllus leaves from shoots cultured in vitro lacked cuticular waxes, had a higher water content and consisted mainly of spongy parenchyma cells. Leshem (62) also observed changes in the internal structure of vitreous carnation shoots and leaves. Two recent articles concerning plants propagated in vitro reported on the presence of large intercellular air spaces in the mesophyll of Rubus idaeus leaves (29) and Picea abies needles (8). These anatomical changes and the waterlogged appearance of vitreous tissues and organs may provide clues to the mechanisms responsible for vitrification.

Accumulation of ethylene is one of the first responses to the onset of waterlogging (13, 52) and ethylene is known to be the agent responsible for many of the injury symptoms associated with waterlogging (13, 51, 52) including: guttation, intumenescence formation and stem hypertrophy (4). Interestingly, each of these symptoms was observed in vitreous A. rubrum 'Red Sunset' cultures. Another symptom of waterlogging is aerenchyma formation. These are large intercellular air spaces which occur in the roots and stems of aquatic plants and to some extent in other plants as an adaptation to waterlogging (52). There are two types of aerenchyma: lysigenous and schizogenous. The first type is formed by the disintegration of entire cells, while the second results from the separation of cell walls from each other (52). Kawase (52) suggested that a sequence of events lead to aerenchyma formation: first, waterlogging followed by ethylene production which stimulates increased cellulase activity and induces aerenchyma development. These structures allow plants to survive under short term waterlogging, but may not prevent plants from senescing during prolonged flooding. The

structures then become evidence that waterlogging has occurred (52).

Although these observations are circumstantial, they suggest that ethylene accumulation *in vitro* may be responsible for the development of vitreous 'Red Sunset' shoots, especially since *A. rubrum* is known to be tolerant to waterlogging (52).

Recently, Kevers et al. (53) also suggested that ethylene plays a role in vitrification. They concluded that vitrification results from a burst of ethylene controlled by the peroxidase-IAA-oxidase system.

To develop appropriate measures for controlling vitreous shoot formation in 'Red Sunset' shoot tip cultures, a number of factors including: agar, sucrose, cytokinin, and salt concentration; temperature regime, type culture vessel and container volume were examined. The effect which these factors had on shoot proliferation and vitrification will be considered below.

The water potential of the culture medium is a critical factor in the occurrence of vitreous shoots *in vitro* (27, 62, 122). It was possible to eliminate vitrification in 'Red Sunset' cultures by increasing the agar concentration to 12 gl^{-1}. Other researchers have made similar observations (8, 27, 62, 122). Debergh et al. (27) suggested that agar was responsible for the matric potential of the medium water potential and that this component was responsible for vitrification. The key to this observation was that agar does not penetrate plant tissue. Debergh et al. (27) concluded that increasing the agar concentration reduced the availability of cytokinins in the medium. What seems more plausible, however, is that raising the agar concentration creates a slight water stress (62). Such stress may reduce the growth rate of effected tissues and simultaneously reduce vitrification. This hypothesis is supported by the observation that

shoot proliferation rates were significantly reduced in A. rubrum 'Red Sunset' cultures when the agar concentration was above 8 gl^{-1}. Agar concentrations below 8 gl^{-1} also reduced proliferation rates but did not reduce vitrification. This was possible due to excess free water present in the culture medium.

It is interesting to note that increasing the sucrose concentration in 'Red Sunset' culture medium did not reduce vitrification. Debergh et al. (27) made the same observation with Cynara scolymus cultures. They concluded that sucrose and mannitol effect the osmotic potential of the medium water potential, but since these are tissue penetrating, they do not reduce vitrification. The response, however, may be concentration dependant. By raising the sucrose concentration to 8%, Ziv et al. (122) were able to increase the number of normal Dianthus caryophyllus shoots to 93% compared to 54% at 3% sucrose.

Sucrose concentrations below 30 gl^{-1} significantly reduced shoot proliferation and prevented the formation of vitreous shoots 'Red Sunset'. In culture, sucrose acts as a carbon source and when surplus carbon is available also acts as an osmoticum (16). The data suggests that with 10 or 20 gl^{-1} sucrose there was not sufficient carbon to stimulate active growth.

The role which cytokinins play in stimulating the development of vitreous shoots is a matter of speculation. Debergh et al. (27) conducted experiments with Cynara scolymus cultures to determine if vitrification was due to the acculuation of breakdown products of synthetic cytokinins. However, after three culture periods on medium lacking cytokinins, losses due to vitrification were still greater than 50%. In this instance, however, a high level of vitrification was observed at all stages of development in vitro so it would be difficult

to determine if the treatment was having any effect. Cytokinins did, however, play an indirect role in the formation of vitreous 'Red Sunset' shoots. Levels of BA or 2ip, 5.0 or 10.0 mgl^{-1} respectively, which stimulated rapid growth and shoot proliferation also led to the formation of vitreous shoots. Interestingly, vitreous shoots were rarely observed at levels of BA, 2ip and Kinetin which did not support active shoot proliferation. Transferring 'Red Sunset' shoots to basal medium lacking cytokinins also eliminated vitreous shoot formation. These shoots rooted and grew slowly with little or no shoot growth.

'Red Sunset' shoots were very sensitive to reductions in the levels of major and minor salts in the LS medium. However, it was not possible to determine if lowering the salt concentration had any effect on vitrification since the frequency of vitreous shoots was low in all treatments. In any event, this would not be an acceptable method to prevent vitrification because of the drastic reduction in shoot proliferation rates that occurred at lower salt concentrations. It is quite probable that the reduced growth was responsible for the lack of shoot proliferation and vitrification. It is interesting to note, however, that the first developmental response to diminish as the salt level was reduced was shoot proliferation while shoot elongation and callus growth were not significantly restricted until the salt levels were reduced to 50 and 25% respectively.

It was possible to prevent vitreous shoot formation in 'Red Sunset' shoot tips cultures by holding the cultures for four weeks at 20° C followed by two weeks at 25° C. However, this was not a practical method for controlling vitrification since it prevented axillary shoot proliferation. The effect of the 20° C treatment was only temporary. This was quite apparent with cultures given the two week 20° C treatment

and then grown for four weeks at 25° C. Forty-three percent of the shoots harvested from these cultures were vitreous.

Debergh et al. (27) also concluded that a cold treatment (4° C) was not an effective method of controlling vitrification. Their results, however, may not support this conclusion. They reported that Cynara scolymus cultures given the cold treatment were necrotic at the end of 3-4 week culture period but did not indicate the percentage of vitreous shoots.

Condensation of water inside the culture vessel can lead to the development of vitreous shoots (122). Ziv et al. (122) reported that Dianthus caryophyllus shoot tips cultured on a horizontal agar surface where water accumulated were more prone to injury than those cultured on an agar slant. Damage due to water condensation was also evident in 'Red Sunset' cultures. This was especially apparent in 25 x 150 mm culture tubes where shoots growing against the glass frequently became watersoaked and glassy. It was possible to reduce the frequency of vitreous shoots by placing 'Red Sunset' shoot tips in 60 ml bottles. Changing culture vessels also improved axillary shoot quality.

Previously, it was implied that there was a relationship between the growth rate of 'Red Sunset' shoot tips and the degree of vitrification. Thus, reducing the growth rate may reduce vitrification. While this appeared to be the case with respect to agar, cytokinin and salt concentrations and temperature it did not seem to occur with respect to container volume. The rate of shoot proliferation and shoot elongation were reduced in 240 ml bottles campared to 60 ml bottles, but the number of vitreous shoots was higher. These seemingly contradictory results may be explained in the following manner. Shoots placed in the 240 ml bottles grew slower than those in the 60 ml bottles during the

initial four week culture period possibly due to lower relative humidity in the larger vessels. However, decapitation and transfer to fresh medium stimulated shoot development and excessive basal callus in the 240 ml bottles compared to the 60 ml bottles. It is possible that the movement of nutrients across the callus/medium interface led to the development of positive root pressure. Interestingly, guttation, a symptom of positive root pressure which occurs under conditions of high humidity (78), was frequently associated with injured 'Red Sunset' shoot tips suggesting that the large basal callus mass stimulated vitrification by allowing excess water to enter the explants.

Debergh et al. (27) also studied the effects of container volume on the development of vitreous shoots and conlcuded that vitrification was not due to the presence of volatile compounds in the atmosphere. This conclusion, however, may not be warranted by their experimental design and lack of supporting evidence. First, liquid medium as used in their experiments has subsequently been shown to stimulate vitrification (53, 122). Second, they did not analyze gas samples to determine the presence of possible deleterious volatile compounds. These arguements are important since Kever et al. (53) reported that explants placed in liquid medium immediately produced more ethylene than those placed on solid medium.

Control of vitrification in 'Red Sunset' shoot tip cultures was complicated by the fact that most treatments which reduced or prevented the formation of vitreous cultures also reduced shoot proliferation rates. While this situation may appear irreversible, in some cases, it has been possible to successfully control vitrification and maintain acceptable shoot proliferation by modifying culture techniques. For example, Ziv et al.(122) proposed two possible solutions for controlling

vitrification in Dianthus caryophyllus cultures: 1) place shoots in liquid medium for a few days and then transfer to agar solidified medium or 2) place shoots on agar slants and later transfer uncapped cultures to a desiccator. Both of these proposals worked on the principle that it was possible to stimulate active shoot proliferation and then control vitrification. A similar suggestion was made for Picea abies cultures (8). Using the same principle, it was possible to control vitrification in decapitated 'Red Sunset' cultures by harvesting shoots before damage was observed. Culturing shoots in 60 ml bottles rather than 25 x 150 mm culture tubes also reduced the incidence of vitreous shoots. It may also be possible to reduce vitrification in 'Red Sunset' cultures by reducing the relative humidity in vitro as suggested by Ziv et al. (122), although placing cultures in a desiccator as they suggested seems rather impractical on a commercial scale. Use of a closure which would allow water vapor to escape may overcome this problem.

Exogenous auxins were not required to stimulate rooting of A. rubrum 'Red Sunset' shoot tips in vitro. Proliferated shoots rooted readily in culture tubes and in 0.95 liter jars. One possible explanation for this ease of rooting is that 'Red Sunset' shoots contain a high level of endogenous auxin. The same argument was used to explain the ease of rooting of Lycopersicon esculentum 'Star Fire' (50) and Browallia viscosa (114) shoots in culture. Support for this hypothesis with 'Red Sunset' comes indirectly from two sources: 1) high levels of cytokinin were required to overcome apical dominance and 2) removing the source of endogenous auxins by decapitation allowed rapid shoot proliferation.

Another explanation for the ease of rooting of 'Red Sunset' shoot tips is the phenomenon of rejuvenation. Skiskandarajah and Mullins (93)

suggested that culture techniques lead to physiological rejuvenation. Earlier, Welsh et al. (115) concluded that rooting of Red Maple shoots *in vitro* was closely linked to their physiological state. Thus, conditions stimulating active growth and shoot proliferation would also enhance rooting. Changes in the physiological state of the explants in culture have been reported for a number of genera including: *Malus* (93), *Rosa* (41), *Vaccinium* (65, 66) and *Vitis* (9, 90).

With some species, for example, *Vaccinium* (65, 66) and *Vitis* (9, 90), rejuventated shoots bore distinctly juvenile morphological characteristics. Lyrene (65) described two types of blueberry shoots: 1) shoots with large leaves and heavy stems resembling mature growth and 2) juvenile appearing shoots with small leaves and filamentous stems. The phenotypic juvenile shoots rooted more readily than seedling material (66). Morphological differences were also observed between 'Red Sunset' plants recovered from culture and mature, field grown trees. The leaves of the trees propagated *in vitro* were generally five-lobed and cordate at the base while the leaves of the field grown trees were three-lobed and rounded at the base. Similar observations were made, however, between grafted 'Red Sunset' trees of different ages, suggesting that these differences in leaf shape may only reflect the growth rate of the trees.

The ease of rooting of 'Red Sunset' shoots has another implication. An essential feature of an economically feasible micropropagation system is the ability to root a high percentage of shoots enmasse over a relatively short period of time. This has been demonstrated with 'Red Sunset'. It may also be possible to root 'Red Sunset' shoots under septic conditions in a high humidity chamber. This approach has been used successfully with *Kalmia* (63) and *Betula* (69) microcuttings.

The uniformity of 'Red Sunset' plants propagated *in vitro* is an indication that this cultivar is stable genetically. This means that 'Red Sunset' cultures can be maintained for long periods of time without needing to start new cultures on an annual basis and go through the initial problems involved. It also indicates that the axillary shoot proliferation system employed here is a relatively safe method for propagating plants *in vitro*. Others (41, 63, 67, 69, 76) have made the same observation.

SUMMARY

Aseptic, actively growing cultures of Acer rubrum 'Red Sunset' were established using a preconditioning treatment which consisted of transferring shoot tip or node segment explants, 3-4 cm , at 3-4 day intervals to fresh LS basal medium or with 1.0 mgl^{-1} BA or 2ip. This procedure made it possible to select actively growing explants and to discard contaminated and phenol damaged tissues. Holding surface sterilized 'Red Sunset' explants in a humid environment prior to dissection also helped to reduce injury by polyphenols.

The most effective cytokinin in stimulating axillary shoot proliferation in 'Red Sunset' shoot tip cultures was BA with 5.9 shoots/explant at 5.0 mgl^{-1} followed by 2ip with 3.0 shoots/explant at 10.0 mgl^{-1}. It was suggested that the high level of cytokinin required to stimulate maximum shoot proliferation was possibly due to high levels of endogenous auxin in the 'Red Sunset' shoots in vitro. This hypothesis was partially supported by the observation that shoot proliferation was most consistent following decapitation.

Axillary shoot proliferation was also stimulated by the incorporation of 100 mgl^{-1} adenine sulfate in the LS + 10.0 mgl^{-1} 2ip multiplication medium and this concentration also significantly reduced basal callus fresh weight. These observations were interesting because they implied that excess basal callus had a negative effect on shoot proliferation.

Conditions in vitro which stimulated optimum shoot proliferation in

'Red Sunset' cultures including: high levels of BA or 2ip, high salt levels, adequate sucrose concentration, readily available free water and proper temperature regime also led to the formation of vitreous shoots. It was possible to control vitrification in decapitated 'Red Sunset' cultures by harvesting shoots before damage was observed. Culturing shoots in 60 ml bottles rather than 25 x 150 mm culture tubes also reduced the incidence of vitreous shoots.

No exogenous auxins were required to stimulate rooting of 'Red Sunset' shoots *in vitro*. This observation agrees with the hypothesis proposed earlier that 'Red Sunset' shoots contain a high level of endogenous auxin(s). The ease of rooting of 'Red Sunset' shoots *in vitro* could also be explained by the phenomenon of rejuvenation.

With few exceptions, plants recovered from culture were uniform phenotypically. This was an indication that 'Red Sunset' is genetically stable and that this cultivar can be safely propagated *in vitro* using an axillary shoot proliferation system.

Several areas require additional research: 1) the protocol described above should be extended to other cultivars, 2) plants propagated *in vitro* should be evalutate to determine their performance and phenotypic stability under field conditions and 3) efforts should be made to gain a better understanding of factors responsible for vitrification, and to develop suitable control measures.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Abbott, A. J. 1977. Propagating temperate woody species in tissue culture. Scientia Hort. 28: 155-162.

2. Abbott, A. J. 1978. Practice and promise of micropropagation of woody species. Acta. Hort. 79: 113-127.

3. Abbott, A. J and E. Whiteley. 1976. Culture of Malus tissues in vitro. I. Multiplication of apple plants from isolated shoot apices. Scientia Hort. 4: 183-199.

4. Abeles, F. B. 1973. Ethylene in plant biology. Academic Press, New York.

5. Aitken, J., K. J. Horgan and T. A. Thorpe. 1981. Influence of explant selection on the shoot-formation capacity of juvenile tissue of Pinus radiata. Can. J. For. Res. 11: 112-117.

6. Anderson, W. C. 1975. Propagation of rhododendrons by tissue culture: Part 1. Development of a culture medium for multiplication of shoots. Proc. Inter. Plant Prop. Soc. 25: 129-135.

7. Anderson, W. C. 1980. Mass propagation by tissue culture: Principles and practice, p. 1-10. Proceedings of the Conference of Nursery Production of Fruit Plants Through Tissue Culture--Appilcations and Feasability. USDA, Beltsville.

8. Arnold, S. von and T. Eriksson. 1981. Effect of agar concentration on growth and anatonomy of adventious shoots of Picea abies (L.) Karst. Plant Cell Tissue Organ Culture 3: 257-264.

9. Barlass, M. and K. G. M. Skene. 1980. Studies on the fragmented shoot apex of grapevine. II. Factors affecting growth and diferentaion in vitro. J. Exp/ Bot. 31: 489-495.

10. Ben-Jaacov, J. and E. Dax. 1981. In vitro propagation of Grevillea rosmarinifolia. HortScience 16: 309-310.

11. Biondi, S. and T. A. Thorpe. 1981. Requirements for a tissue culture facility, p. 1-20. In: T. A. Thorpe (ed.). Plant tissue culture methods and applications in agriculture. Academic Press, New York.

12. Borkowska, B. and L. E. Powell. 1979. The dormancy status of

apple buds determined by an *in vitro* culture system. J. Amer. Soc. Hort. Sci. 104: 796-799.

13. Bradford, K. J. and S. F. Yang. 1981. Physiological responses of plants to waterlogging. HortScience 16: 25-30.

14. Broome, O. C. and R. H. Zimmerman. 1978. *In vitro* propagation of blackberry. HortScience 13: 151-153.

15. Brown, D. M., C. L. Groom, M. Cvitanik, M. Brown, J. L. Cooper and J. Arditti. 1982. Effects of fungicides and bactericides on orchid seed germination and shoot tip cultures *in vitro*. Plant Cell Tissue Organ Culture 1: 165-180.

16. Brown, D. W. C., D. W. M. Leung and T. A. Thorpe. 1979. Osmotic requirement for shoot formation in tobacco callus. Physiol. Plant. 46: 36-41.

17. Buchelard, E. P. and B. B. Stowe. 1963. Rooting of cuttings of *Acer rubrum* L. and *Eucalyptus camaldulensis* Dehn. Aust. J. Biol. Sciences 16: 751-767.

18. Chalupa, V. 1974. Control of root and shoot formation and production of trees from poplar callus. Biol. Plant. 16: 316-320.

19. Chalupa, V. 1981. *In vitro* propagation of birch (*Betula verrucosa*). Biol. Plant. 23: 472-474.

20. Chapman, D. J. 1979. Propagation of *Acer campestre*, *A. platanoides*, *A. rubrum*, and *A. ginnala* by cuttings. Proc. Inter. Plant Prop. Soc. 29: 345-348.

21. Chaturvedi, H. C., A. K. Sharma and R. N. Prasad. 1978. Shoot apex culture of *Bougainvillea glabra* 'Magnifica'. HortScience 13: 36-37.

22. Cheng, T. Y. 1978. Clonal propagation of woody plant species through tissue culture techniques. Proc. Inter. Plant Prop. Soc. 28: 139-155.

23. Christie, C. B. 1978. Rapid propagation of aspens and silver poplars using tissue culture techniques. Proc. Inter. Plant Prop. Soc. 28: 255-260.

24. Clark, J. R. 1981. Juvenility and plant propagation. Proc. Inter. Plant Prop. Soc. 31: 449-453.

25. Cohen, D. 1980. Application of micropropagation methods for blueberries and tamarillos. Proc. Inter. Plant Prop. Soc. 30: 144-146.

26. Davies, D. R. 1980. Rapid propagation of roses *in vitro*. Scientia Hort. 13: 385-389.

27. Debergh, P., Y. Harbaoui and R. Lemeur. 1981. Mass propagation of globe artichoke (Cynara scolymus): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53: 181-187.

28. Dirr, M. A. 1977. Manual of woody landscape plants: Their identification, ornamental characteristics, culture, propagation and uses. Stipes Publishing Co., Champaign, Illinois.

29. Donnelly, D. J. and W. E. Vidaver. 1984. Leaf anatomy of red raspberry transferred from culture to soil. J. Amer. Soc. Hort. Sci. 109: 172-176.

30. Edgerton, L. J. 1944. Two factors affecting rooting of red maple cuttings. J. For. 42: 678-679.

31. English, J. A. 1981. Rooting Acer rubrum cultivars using single node cuttings. Proc. Inter. Plant Prop. Soc. 31: 147-148.

32. Fari, M. and M. Czako. 1981. Relationship between position and morphogenetic response of pepper hypocotyl explants cultured in vitro. Scientia Hort. 15: 207-213.

33. Fordham, I., D. P. Stimart and R. H. Zimmerman. 1982. Axillary and adventitous shoot proliferation of Exbury azaleas in vitro. HortScience 17: 738-739.

34. Garland, P. and L. P. Stoltz. 1981. Micropropagation of Pissardi plum. Ann. Bot. 48: 387-389.

35. Garton, S., M. A. Hosier, P. E. Read and R. S. Farnham. 1981. In vitro propagation of Alnus glutinosa Gaertn. HortScience 16: 758-759.

36. Gorst, J. R., R. A. Bourne, S. E. Hardaker, A. E. Richards, S. Dircks and R. A. de Fossard. 1978. Tissue culture propagation of two Grevillea hybrids. Proc. Inter. Plant Prop. Soc. 28: 435-446.

37. Hammerschlag F. 1982a. Factors affecting establishment and growth of peach shoots in vitro. HortScience 17: 85-86.

38. Hammerschlag F. 1982b. Factors influencing in vitro multiplication and rooting of the plum rootstock Myrobalan (Prunus cerasifera Ehrh.). J. Amer. Soc. Hort. Sci. 107: 44-47.

39. Harris, R. E. and J. H. Stevenson. 1982. In vitro propagation of Vitis. Vitis 21: 22-32.

40. Hasegawa, P. M. 1979. In vitro propagation of rose. HortScience 14: 610-612.

41. Hasegawa, P. M. 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. J. Amer. Soc. Hort. Sci. 105: 216-220.

42. Helgeson, J. P. 1968. The cytokinins. Science 161: 974-981.

43. Hu, C. Y. and P. J. Wang. 1983. Mersitem, shoot tip and bud cultures, p. 177-227. In: D. A. Evans, W. R. Sharp, P. V. Ammirato and Y. Yamada (eds.). Handbook of plant cell culture. Volume 1. MacMillan Publishing Co., New York.

44. Hughes, K. W. 1981. Ornamental species, p. 5-50. In: B. V. Conger (ed.). Cloning agricultural plants via in vitro techniques. CRC Press, Inc., Boca Raton, Florida.

45. Huhtinen, O. 1976. Early flowering of birch and its maintenance in plants regenerated through tissue culture. Acta. Hort. 56: 243-247.

46. Hyndman, S. E., P. M. Hasegawa and R. A. Bressan. 1982. Stimulation of root initiation from cultured rose shoots through the use of reduced concentrations of mineral salts. HortScience 17: 82-83.

47. Jones, O. P. 1976. Effect of phloridzin and phloroglucinol on apple shoots. Nature. 262: 392-393.

48. Jones, O. P. and M. E. Hopgood. 1979. The successful propagation in vitro of two rootstocks of Prunus: the plum rootstock, Pixy (P. insititia) and the cherry rootstock F 12/1 (P. avium). J. Hort. Sci. 54: 63-66.

49. Jones, O. P., M. E. Hopgood and D. O'Farrell. 1977. Propagation in vitro of 'M26' apple rootstock. J. Hort. Sci. 52: 235-238.

50. Kartha, K. K., O. L. Gamborg, J. P. Shyluk and F. Constabel. 1976. Morphogenetic investigations on in vitro leaf culture of tomato (Lycopersicon esculentum Mill. cv. Starfire) and high frequency plant cel generation. Z. Pflanzenphysiol. 77: 292-301.

51. Kawase, M. 1976. Ethylene accumulation in flooded plants. Physiol. Plant. 36: 236-241.

52. Kawase, M. 1981. Anatomical and morphological adaptations of plants to waterlogging. HortScience 16: 30-34.

53. Kevers, C., M. Coumans, M. -F. Coumans-Gilles and Th. Gaspar. 1984. Physiological and biochemical events leading to vitrification of plants cultured in vitro. Physiol. Plant. 61: 69-74.

54. Kosh-Khui, M. aand K. C. Sink. 1982. Micropropagation of new and old world rose species. J. Hort. Sci. 57: 315-319.

55. Kozel, P. C. and M. Jansen. 1976. A new look at red maple. Amer. Nurseryman 143(12): 7, 70-72 and 83.

56. Krul, W. R. and J. Myerson. 1980. In vitro propagation of grape, p. 35-43. Proceedings of the Conference on Nursery Production of Fruit Plants Through Tissue Culture--Applications and Feasability. USDA, Belstville.

57. Kyte, L. and B. Briggs. 1979. A simlpified entry into tissue culture production of rhododendrons. Proc. Inter Plant Prop. Soc. 29: 90-95.

58. Lane, B. H. and S. Still. 1981. Influence of extended photoperiod ad fertilization on rooting Acer rubrum L. 'Red Sunset' cuttings. Proc. Inter. Plant Prop. Soc. 31: 571-578.

59. Lane, W. D. 1979a. In vitro propagation of Spirea bumalda and Prunus cisterna from ghost apices. Can. J. Plant Sci. 59: 1025-1030.

60. Lane, W. D. 1979b. Regeneration of pear plants from shoot meristem-tips. Plant Sci. Lett. 16: 337-342.

61. Leopold A. C. and P. E. Kriedemann. 1975. Plant growth and development. 2nd Ed. McGraw-Hill Book Co., New York.

62. Leshem, B. 1983. Growth of carnation meristems in vitro: Anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. Ann. Bot. 52: 413-415.

63. Lloyd, G. and B. McCown. 1980. Commercially-feasible micropropagation of Mountain Laurel, Kalmia latifolia, by use of shoot-tip culture. Proc. Inter. Plant Prop. Soc. 30: 421-427.

64. Lundergran, C. A. and J. Janick. 1980. Regulation of apple shoot proliferation and growth in vitro. Hort. Res. 20: 19-24.

65. Lyrene, P. M. 1980. Micropropagation of rabbiteye blueberries. HortScience 15: 80-81.

66. Lyrene, P. M. 1981. Juvenility and production of fast-rooting cuttings from blueberry shoot cultures. J. Amer. Soc. Hort. Sci. 106: 396-398.

67. McComb, J. A. 1978. Clonal propagation of woody plants using tissue culture, with special reference to apples. Proc. Inter. Plant Prop. Soc. 28: 413-426.

68. McCown, B. H. 1981. The use of microculture (tissue culture) in the production and improvement of nursery crops, p. 6-8. In: New Horizons from the Horticultural Research Institute

1980. Horticulture Research Institute, Washington, D.C.

69. McCown, B. and R. Amos. 1979. Initial trials with commercial micropropagation of birch selection. Proc. Inter. Plant Prop. Soc. 29: 387-393.

70. Mehra, A. and P. N. Merha. 1974. Organogenesis and plantlet formation in vitro in almond. Bot. Gaz. 135: 61-73.

71. Miller, C. O., F. Skoog, M. H. von Saltza and F. M. Strong. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. J. Amer. Chem. Soc. 77: 1392.

72. Miller, C. O., F. Skoog, F. S. Okumura, M. H. von Slatza and F. M. Strong. 1956. Isolation, structure and synthesis of kinetin a substance promoting cell division. J. Amer. Chem. Soc. 78: 1375-1380.

73. Miller, G. A., D. C. Coston, E. G. Denny and M. E. Romeo. 1982. In vitro propagation of Nemaguard peach rootstock. HortScience 17: 194.

74. Mott, R. L. 1981. Trees, p. 217-254. In: B. V. Conger (ed.). Cloning agricultural plants via in vitro techniques. CRC Press, Inc. Boca Raton, Florida.

75. Murashige, T. 1974. Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25: 135-166.

76. Murashige, T. 1977. Manipulation of organ initiation in plant tissue cultures. Bot. Bull. Academia Sinica. 18: 1-24.

77. Murashige, T. 1978. The impact of plant tissue culture on agriculture, p. 15-26. In: T. A. Thorpe (ed.). Frontiers of plant tissue culture. University of Calgary, Calgary, Alberta, Canada.

78. Noggle, G. R. and G. J. Fritz. 1976. Introductory plant physiology. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

79. Norton, M. E. and A. A. Boe. 1982. In vitro propagation of ornamental Rosaceous plants. HortScience 17: 190-191.

80. Oka, S. and K. Ohyuma. 1981. In vitro initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissues of mulberry (Morus alba). Can. J. Bot. 59: 68-74.

81. Orton, E. R., Jr. 1978. Single node cuttings: A simple method for the rapid propagation of plants of selected clones of Acer rubrum L. The Plant Propagator 24: 12-15.

82. Romberger, J. A. and C. A. Tabor. 1971. The Picea abies shoot apical meristem in culture. 1. Agar and autoclaving effects.

Amer. J. Bot. 58: 131-140.

83. Rosati, P., G. Marino and C. Swierczewski. 1980. In vitro propagation of Japanese Plum (Prunus salicina Lindl. cv. Calita). J. Amer. Soc. Hort. Sci. 105: 126-129.

84. Salisbury, F. B. and C. Ross. 1969. Plant physiology. Wadsworth Publishing Co. Inc., Belmont, California.

85. Schwab, B. W. 1979. New techniques for growing west coast trees. Amer. Nurseryman 149(9): 70-72.

86. Sharma, A. K., R. N. Prasad and H. C. Chaturvedi. 1981. Clonal propagation of Bougainvillea glabra 'Magnifica' through shoot apex culture. Plant Cell Tissue Organ Culture 1: 33-38.

87. Singha, S. 1982a. In vitro propagation of crabapple cultivars. HortScience 17: 191.

88. Singha, S. 1982b. Influence of agar concentration on in vitro shoot proliferation of Malus sp. 'Almey' and Pyrus communis 'Seckel'. J. Amer. Soc. Hort. Sci. 107: 657-660.

89. Sink, K. C. and J. B. Power. 1977. The isolation, culture and regeneration of leaf protoplasts of Petunia parviflora Juss. Plant Sci Lett. 10: 335-340.

90. Skene, K. G. M. and M. Barlass. 1980. Micropropagation of grapevine. Proc. Inter. Plant Prop. Soc. 30: 564-570.

91. Skirvin, R. M. 1981. Fruit crops, p. 51-139. In: B. V. Conger (ed.). Cloning agricultural plants via in vitro techniques. CRC Press, Inc. Boca Raton, Florida.

92. Skirvin, R. M. and M. C. Chu. 1979. In vitro propagation of 'Forever Yours' rose. HortScience 14: 608-610.

93. Skiskandarajah, S. and M. G. Mullins. 1981. Micropropagation of apple scion cultivars. Proc. Inter Plant Prop. Soc. 31: 209-213.

94. Skoog, F. and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tisues cultured in vitro. Symp. Soc. Exp. Biol. 11: 118-131.

95. Smith, H., E. E. Billett and A. B. Giles. 1977. The photocontrol of gene expression in higher plants, p. 95-127. In: H. Smith (ed.). Regulation of enzyme synthesis and activity in higher plants. Academic Press, New York.

96. Snir, I. 1982. In vitro propagation of sweet cherry cultivars. HortScience 17: 192.

97. Snow, A. G., Jr. 1941. Variables affecting vegetative propagation of red and sugar maple. J. For. 39: 395-404.

98. Srivastava, P. V. and A. Steinhauer. 1981. Regeneration of birch plants from catkin tissue cultures. Plant Sci. Lett. 22: 379-386.

99. Stokes, M. J. 1980. Current aspects of commercial micropropagation. Proc. Inter. Plant Prop. Soc. 30: 255-267.

100. Strode, R. E., P. A. Travers and R. P. Oglesby. 1979. Commercial micropropagation of rhododendrons. Proc. Inter. Plant Prop. Soc. 29: 439-443.

101. Sutter, E. and R. W. Langhans. 1979. Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Amer. Soc. Hort. Sci. 104: 493-496.

102. Swartz, H. J., G. J. Galletta and R. H. Zimmerman. 1981. Field performance and phenotypic stability of tissue culture-propagated strawberries. J. Amer. Soc. Hort. Sci. 106: 667-673.

103. Tabachnik, L. and D. E. Kester. 1977. Shoot cultures for almond-peach hybrid clones in vitro. HortScience 12: 545-547.

104. Ting, I. P. 1982. Plant physiology. Addison-Wesley Publishing Co. Inc., Philippines.

105. Torrey, J. G. 1977. Cytodifferentiation in cultured cells and tissues. HortScience. 12: 14-15.

106. Townsend, A. M. 1977. Charactersistics of red maple progenies from different geographical areas. J. Amer. Soc. Hort. Sci. 102: 461-466.

107. Townsend, A. M. 1979. An analysis of red maple characteristics. Amer. Nurseryman 150(9): 12, 13, 66, 68, 70.

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

109. Waithaka, K., A. C. Hildebrant and M. N. Dana. 1980. Hormonal control of strawberry axillary bud development in vitro. J. Amer. Soc. Hort. Sci. 105: 428-430.

110. Walkey, D. G. 1972. Produdution of apple plantlets from axillary bud meristems. Can. J. Plant Sci. 52: 1085-1087.

111. Wardle, K., E. B. Dobbs and K. C. Short. 1983. In vitro acclimatization of aseptically cultured plantlets to humidity. J. Amer. Soc. Hort. Sci. 108: 386-389.