SHORT TITLE

MICROPROPAGATION OF *MALUS*

ENG-CHONG PUA

ABSTRACT

Ph.D ENG-CHONG PUA Plant Science (Horticulture)

MORPHOGENESIS AND PHYSIOLOGY OF MALUS TISSUES AND CELLS IN VITRO

Three apple genotypes, Ottawa 3, Malus robusta No. 5, and Macspur (juvenile and adult phases) were successfully propagated in vitro. Normal shoot cultures of Ottawa 3 were obtained only on sucrose medium containing combinations of benzyladenine, naphthaleneacetic acid or indolebutyric acid (IBA), and gibberellic acid. Sorbitol was a prerequisite for growth of meristem tip explants and was most beneficial for shoot proliferation of M. robusta. Juvenile phase Macspur cultures maintained on sucrose medium grew equally well when subcultured on sorbitol and sucrose media, and showed a significantly higher shoot number than corresponding adult phase cultures; shoot proliferation of adult phase cultures was best on sorbitol medium.

The best rooting response for apple genotypes was obtained on agar medium containing 6.25 mg/L IBA and sucrose for Ottawa 3 (79%); 0.5 mg/L IBA and either sorbitol or sucrose for M. robusta (85%); 0.25 mg/L IBA and either sorbitol or sucrose for juvenile phase (71-81%) and 3.0 mg/L IBA and sucrose for adult phase Macspur.

Induction of somatic embryogenesis in cell suspension cultures was not successful.

RESUME

Ph.D. ENG-CHONG PUA Plant Science (Horticulture)

MORPHOGÉNÈSE ET PHYSIOLOGIE DES CELLULES ET DES TISSUS DE MALUS EN CULTURE IN VITRO

La culture in vitro de trois génotypes de pommiers, Ottawa 3, Malus robusta No. 5, et Macspur (phase juvénile et adulte) a été réalisée. La croissance normale de la tige d'Ottawa 3 n'est obtenue que sur milieu au sucrose contenant un mélange de benzyladénine, d'acide naphthalèneacétique ou d'acide indolebutyrique (AIB) et d'acide gibbérellique. Le sorbitol est essentiel à la croissance des explantats de points végétatifs et stimule à un niveau significatif la prolifération des tiges de M. robusta. Les tissus de Macspur en phase juvénile entretenus sur milieu au sucrose, lorsque repiqués, se développent aussi bien sur milieu au sucrose que sur milieu au sorbitol, et produisent un plus grand nombre de tiges que les tissus en phase adulte. La prolifération des tiges dans les cultures des tissus en phase adulte est la plus forte sur milieu au sorbitol.

Le meilleur développement de racines dans les cultures des différents génotypes est obtenu sur les milieux contenant: 6,25 mg/L AIB et sucrose pour Ottawa 3 (79%), 0,5 mg/L AIB et sorbitol ou sucrose pour M. robusta (85%), 0,25 mg/L AIB et sorbitol ou sucrose pour la phase juvénile (71-81%) et 3,0 mg/L AIB et sucrose pour la phase adulte de Macspur entretenue sur milieu au sucrose.

Le déclenchement de l'embryogénèse somatique dans les cultures de cellules en suspension n'a pas été réussi.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
AC	activated charcoal
ad	adenine
BA	benzyladenine
ca	(circa) approximately
CC	cytoplasmic cell
CFI	callus formation index
CI	compactness index
CM	coconut milk
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
DNH	Dudit, Nemeth, and Haydu
DP	Dutcher and Powell
EC	Enzyme Commission number
F6P	fructose-6-phosphate
$FeNA_2EDTA$	ethylenediaminetetraacetic acid, iron (III) derivatives, sodium salts (13% iron)
G6P	glucose-6-phosphate
GA	gibberellic acid
GI	growth index
glu	glutamate
gly	glycine
GMO	Gamborg, Miller, and Ojima
IAA	indoleacetic acid
IBA	indolebutyric acid
ino	inositol
2ip	N^6(Δ-isopentenyl)-amino purine
klux	kilolux
LNI	leaf normality index
LS	Linsmaire and Skoog
Mg	magnessium
mino	myo-inositol
MS	Murashige and Skoog
NAA	naphthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

PCV	packed cell volume
pg	phloroglucinol
pvp	polyvinylpyrolidone
RNA	ribonucleic acid
S1P	sorbitol-1-phosphate
S6P	sorbitol-6-phosphate
S6PD	sorbitol-6-phosphate dehydrogenase
SDH	sorbitol dehydrogenase
SH	Shenk and Hildebrandt
SO	sorbitol oxidase
So + Su	sorbitol (%) + sucrose (%)
su	sucrose
th	thiamin.HCl
UM	Uchimiya and Murashige
v/v	volume/volume
VC	vacuolated cell
w/v	weight/volume

CHAPTER I

INTRODUCTION

Current trends toward high-density plantings and interest in the potential use of own-rooted cultivars have created the need for more efficient and rapid propagation methods capable of meeting the increased demand for new apple fruit stocks. Clonal propagation of apple is normally achieved by conventional methods such as stooling (mound layering) and budding or grafting on seedling or vegetatively propagated rootstocks. A study of ultra-high density (Lilliputian) apple planting systems utilizing up to 37,000 trees/acre (90,000 trees/ha) was first reported in Michigan by Bible (1969). Later, in England, Hudson (1971) proposed the high-density "meadow orchard" system utilizing between 30,000 and 100,000 trees/ha; this system was later developed by Child (1972) and Luckwill and Child (1973).

These new planting systems could become economically feasible if

large numbers of inexpensive trees growing on their own roots are available (Abbott and Whiteley 1976). The vegetative propagation of apple by tissue culture methods, which can be conducted in the laboratory all year-round using very little space, and often at low costs, offers the best alternative means for rapid clonal propagation of this species.

Clonal propagation of plants is based on the concept of totipotency in which all single living cells possess the ability to develop into whole plants via the developmental pathway similar to that of a zygote (Raghavan 1979). Considerable success in clonal propagation has been achieved by in vitro culture of differentiated plant organs, such as meristems and shoot tips, but in the long term, plant regeneration through somatic embryogenesis in suspension culture seems to offer even greater potential for mass clonal propagation. However, success in somatic embryogenesis in cell suspension cultures has been limited to herbaceous species (Abbott 1978; Tisserat et al. 1979).

While in vitro methods seem to have unlimited potential in that it is theoretically possible to culture and propagate any plant species, many problems of propagation are peculiar to woody plants, and attempts to solve them using tissue culture have often been disappointing. Knowledge of the physiology and biochemistry of the organ-forming process is very limited; the lack of suitable experimental systems for such studies is a major problem (Thorpe 1980).

Evidence from the literature suggests that the "juvenility factor" could play an important role in morphogenesis in vitro. Cultures derived

from embryos and seedlings show more potential for morphogenesis than those derived from mature sources (Tran Thanh Van 1981). Organs such as roots, and shoots, embryos and/or plantlets have been obtained from juvenile tissues of Pinus, Picea, Thuja, Cryptomeria, Ulmus, Ilex, and Malus (Abbott 1978; Coffin 1974). According to Heuser (1976) and Hartmann and Kester (1983), overwhelming evidence indicates that the fundamental single factor limiting rooting ability of difficult-to-root woody plants by cuttings is the loss of juvenility. Since the effect of the "juvenility factor" may continue to persist in tissue culture, if non-juvenile (mature) stocks are to be used as sources for propagating material, whether by tissue culture techniques or conventional propagation methods, this problem must be understood and overcome.

Plant tissue cultures allow direct comparison of juvenile and adult plants in terms of growth and morphogenesis in response to various cultural factors under defined conditions which are not possible with intact plants. Among cultural factors, carbohydrates are of prime importance since these are utilized as carbon sources for organ formation, a high energy-requiring process, and also act as osmotic agents influencing shoot formation (Thorpe 1980). Previous studies in this laboratory by Chong and Taper (1974a) indicated that callus cultures of apple generally grew well when supplied with sorbitol, sucrose, glucose, and fructose but growth on these carbon sources varied with apple genotype. In a study in which callus was obtained from 11 woody species of the Rosaceae and grown on both sorbitol and sucrose media, callus growth on sucrose medium was better for nine species, and equal for one species of crabapple (Malus pumila var. niedzwetzkyana (Dieck) Schneid.

cv. Geneva); growth was better on sorbitol medium for one species of peach (Prunus persica (L.) Batsch cv. Reliance) (Coffin et al. 1976). However, information on the influence of carbon source on morphogenesis of apple and other woody plants is scarce.

The objectives of this study were (a) to investigate the suitability of tissue culture as an alternative tool in the propagation of selected rootstocks and cultivars of Malus; (b) to investigate the influence of carbon source on the regulation of morphogenesis of Malus cultures derived from juvenile and adult sources; and (c) to attempt to develop an in vitro cell culture technique for mass propagation of Malus via cell suspension culture, an objective that has not previously been accomplished directly for a woody species. Plantlets have been obtained directly in suspension culture of some 15 herbaceous species. However, in woody species, Ulmus americana L., apparently normal plantlets were obtained indirectly only after cells from suspension were plated on agar medium (Durzan and Lopushanski 1975).

CHAPTER II

LITERATURE REVIEW

2.1 Morphogenesis of woody plants *in vitro*

2.1.1 Historical

The plant cell and tissue culture technique has developed into a dynamic and important field of endeavor during the last decade. This technique first conceived with the idea of totipotency of cells (Schleiden 1838; Schwann 1839), was later popularized by Virchow (1858) with his famous aphorism "every cell from a cell". Haberlandt (1902) first attempted to demonstrate the concept of totipotency. He postulated that, under appropriate conditions, cells and tissues cultured *in vitro* would

recapitulate the developmental sequences of normal plant growth.

Much of the early work on plant cell and tissue culture was done with carrot (Daucus carota L.) and tobacco (Nicotiana tabacum L.). Major discoveries, which led to the demonstration of totipotency in higher plant cells, occurred between 1955 and 1965. Skoog and Miller (1957) demonstrated the interaction of auxins and cytokinins on morphogenetic events. In vitro experimental procedures for regenerating whole plants from tissues (Reinert 1958a; 1958b; 1959), cell suspension (Steward et al. 1958), and single cells (Vasil and Hildebrandt 1965a; 1965b) were also documented.

More recently, plant tissue culture has increasingly been utilized in practical aspects of agriculture. Some of these significant applications include rapid clonal propagation of selected species or genotypes (Vasil and Vasil 1980); induction of haploid plants from anther and pollen cultures (Sunderland 1979); somatic hybridization via protoplast culture (Cocking and Riley 1981); induction of somatic cells and tissues and selection of mutants for specific purposes, such as disease resistance and tolerance to salts (Tomes and Swanson 1982). This review of literature, however, deals primarily with the morphogenetic and physiological aspects of tissue cultures in relationship with clonal propagation of woody species, with emphasis on Malus.

2.1.2 Rapid clonal propagation

Propagation of woody species by tissue culture has been considerably more difficult in comparison with herbaceous species. Thus, information pertaining to *in vitro* propagation of woody species is more limited (Murashige 1974), although progress within the past decade has been more rapid (Abbott 1978; Lane 1982; Skirvin 1981; Winton 1978).

The *in vitro* multiplication of plants has advantages over conventional methods. Large numbers of genetically identical plants can be recovered from a single plant in a relatively short period of time. For instance, Jones *et al.* (1977) estimated that a single shoot tip of M.26 apple rootstock could produce more than 60,000 shoots in eight months. Large numbers of plantlets can be maintained *in vitro* in a limited space and, in many instances, pathogen-free stocks can be derived from meristem cultures.

Like many new scientific approaches, early attempts to culture shoot apices of apple *in vitro* showed little or no success (Dutcher and Powell 1972; Elliott 1972; Jones 1967). In 1976, Abbott and Whiteley induced shoot tips of Cox's Orange Pippin apple to multiply approximately 10 times a month but shoots rooted poorly. In the same year, plantlets of M.7 and M.26 apple rootstocks were successfully cultured (Jones 1976). *In vitro* techniques, with minor modifications, have now been extended to many other apple scions cultivars and clonal rootstocks (Jones *et al.* 1979; Lane and McDougald 1982; Snir and Erez 1980; Zimmerman and Broome

1981). At this writing, more than 40 Malus species have been successfully propagated in vitro (Appendix Table 1).

2.1.2.1 Stages of in vitro propagation

Tissue culture propagation usually proceeds through a sequence of steps, usually referred to as Stage I, establishment of cultures under aseptical conditions; Stage II, shoot proliferation (multiplication); and Stage III, induction of rooting and acclimatization of plantlets under greenhouse conditions. These stages were first proposed by Murashige (1974) and are illustrated in Figure 1 in a scheme devised by this writer. Some authors refer to Stage III as rooting (pretransplantation), and acclimatization (transplantation) as Stage IV (Chong 1981; Gerlach 1981; Hartmann and Kester 1983).

Stage I

The procedures of explant surface-sterilization used in fruit crops for establishing aseptic cultures has been reviewed by Skirvin (1981). When contaminants could not be controlled by surface-sterilization techniques, sometimes anti-microbial compounds such as benomyl or antibiotics were added to the culture medium (Forsberg 1969; Skirvin and Larson 1978).

Figure 1. Scheme for in vitro propagation of plants.

Stage I

(A) Explants, i.e. shoot tip, bud, leaf, stem, etc.,

(B) Surface-sterilization of explants: 1. running water; 2. 70% ethanol; 3. sodium hypochlorite solution (0.5-1%) with or without wetting agent; 4. sterile-distilled water. (C) Aseptical dissection. (D) Explant implantation: 5. surface-sterilized explants, i.e. leaf, meristem, shoot tip, etc. 6. preconditioning medium. 7. standard nutrient medium. (E) Resulting cultures: 8. rooted shoot; 9. callus; 10. shoot.

Stage II

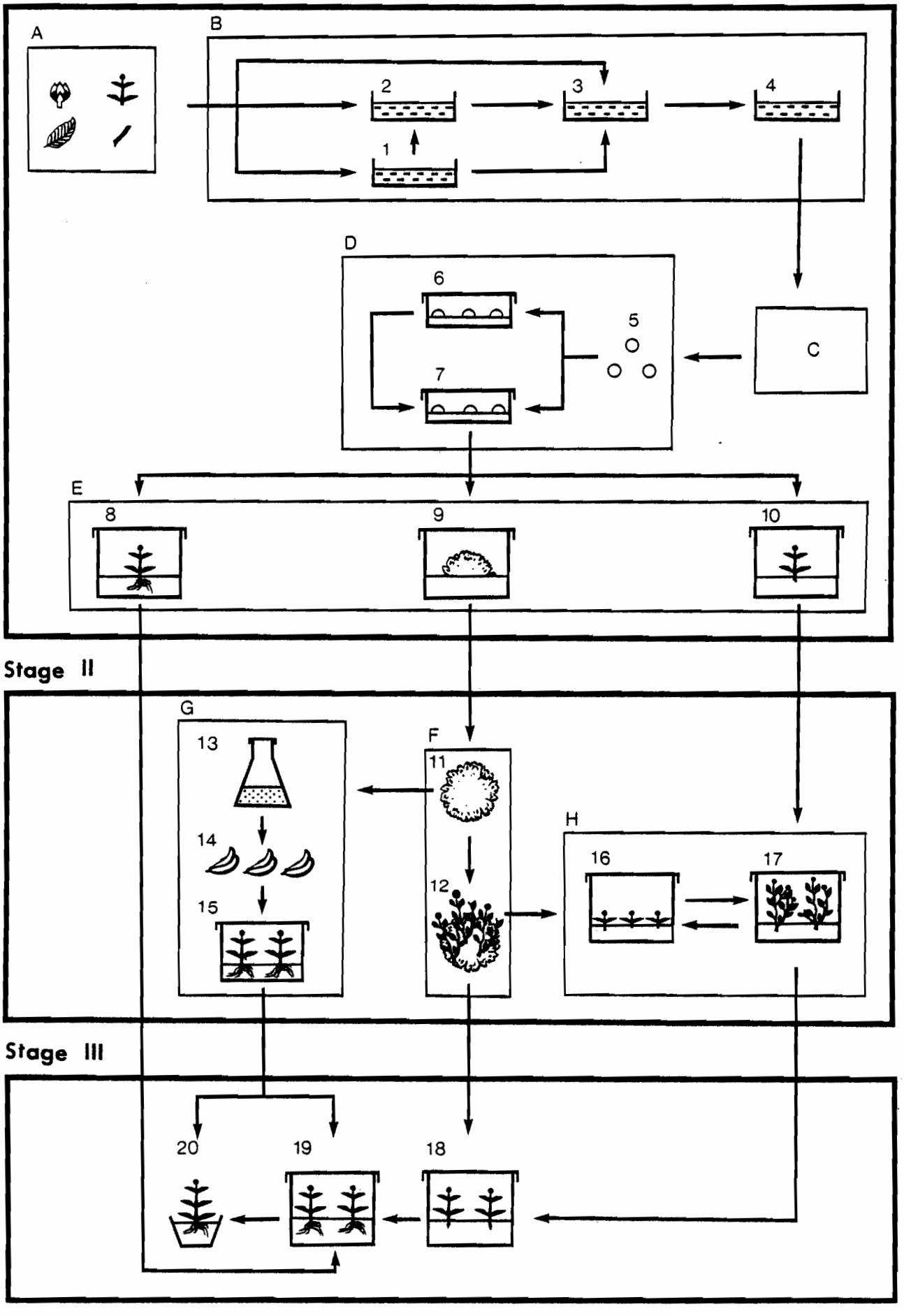
(F) Shoot formation from callus culture: 11. callus; 12. adventitious shoots regenerated from callus

(G) Somatic embryogenesis in cell suspension culture: 13. cell suspension; 14. embryoids; 15. complete plantlets. (H) Shoot multiplication via axillary shoot development: 16. individual shoot tips; 17. cultures with multiple shoots.

Stage III

18. shoot cuttings implanted in rooting medium;

19. rooted shoots; 20. acclimatized plantlets.


Stage I
A
B
2
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4
1
D
6
5
C
7
E
8
9
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Stage II
G
13
F
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H
14
16
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12
15
Stage III
20
19
18

Stage II

Cultures have been maintained on medium containing cytokinin alone (Sriskandarajah et al. 1982), or a combination of cytokinin and auxin (Zimmerman and Broome 1981), for rapid shoot proliferation. Although shoot differentiation from callus has been reported for a number of woody species such as Prunus spp. (Druart 1980), Japanese persimmon (Diospyros kaki Thumb) (Yakoyama and Takenchi 1976), almond (Prunus amygdalus Batch) (Mehra and Mehra 1974), grape (Vitis vinifera L.) (Hirabayashi et al. 1976), and blueberry (Vaccinium angustifolium Ait.) (Lyrene 1978; Zimmerman and Broome 1980), these cultures were not commonly used for mass clonal propagation of fruit crops in view of greater risk of obtaining genetic variants from callus (Murashige 1974).

Stage III

Roots usually are induced by placing individual shoots on the nutrient medium containing auxin such as indolebutyric acid (IBA) or naphthaleneacetic acid (NAA). With apple, half-strength Murashige and Skoog's (MS) (1962) nutrient medium supplemented with either IBA or NAA was commonly used for root induction (Lane 1978; Zimmerman and Broome 1981). High-frequency rooting of apple has been obtained when the rooting medium was supplemented with phloroglucinol (Jones and Hatfield 1976), or activated charcoal (Snir and Erez 1980). Rooting usually was inhibited in the presence of cytokinin (Skirvin 1981). For instance, 85% of shoots of Nemaguard peach were rooted in the presence of NAA alone compared to only 20% in the presence of benzyladenine (BA) (Miller et al. 1982).

85% of shoots of Nemaguard peach were rooted in the presence of NAA alone compared to only 20% in the presence of benzyladenine (BA) (Miller et al. 1982).

During acclimatization, plantlets transplanted to the soil are maintained initially at high humidity, which is gradually reduced. Low percent survival of plants after acclimatization was reported for thornless blackberries (Rubus spp.) (Broome and Zimmerman 1978), rose (Rosa hybrida L.) (Hasegawa 1979; Skirvin and Chu 1979), carnation (Dianthus caryophyllus L.) (Earle and Langhans 1975), and other species. In apple, relatively high percent of plants (85%) survived through this process (Jones et al. 1977). Desiccation and wilting have been the common causes associated with low survival during plant acclimatization (Fuchigami et al. 1981). Evidence indicated that in vitro cultured plants were more vulnerable to desiccation probably due to slow stomatal response to drought (Brainerd and Fuchigami 1981), incomplete vascular system (Grout and Aston 1977), less epicuticular wax (Sutter and Langhans 1979), and smaller palisade cells and mesophyll air space in the leaf compared to greenhouse-grown plants (Brainerd et al. 1981). Cytologically, Wetzstein and Sommer (1982) observed that leaf cells of in vitro cultured, unacclimatized sweetgum (Liquidambar styraciflua L.) plantlets contained large vacuoles, limited cytoplasmic content and flattened chloroplasts with an irregularly arranged internal membrane system in comparison with a greater cytoplasmic content, smaller vacuoles, and well developed chloroplasts in leaf cells of acclimatized or field-grown plants.

2.1.2.2 Tissues and organs

Theoretically, any living plant tissue can be cultured but the degree of success has been variable. In woody plants, the most successful sources (explants) used for in vitro propagation were derived from juvenile material (Skirvin 1981; Sommer and Caldas 1981). Eichholtz et al. (1979a; 1979b) obtained adventitious shoots and embryoids from young apple fruit tissues and speculated that tissues associated with reproduction and seed germination might behave in juvenile-like fashion. In strawberry. adventitious buds were induced from callus of fruit tissue excised either two or seven days, but not 12 days, after anthesis (Ashira and Kano 1977). The superiority of juvenile explants was also demonstrated by Lyrene (1978; 1980) who found that shoot cultures of rabbiteye blueberry (Vaccinium ashei Reade) originating from shoot tips of juvenile plants were easier to establish and culture than those from adult plants.

Variation in growth among explants in vitro was partly attributed to difference in initial explant size (Murashige 1974). Large explants such as shoot tips ($>$ 5 mm) generally are easier to survive in culture than smaller explants such as meristem tips ($<$ 2 mm). Quoirin et al. (1974) found that large shoots of Prunus rooted more easily than small shoots. In meristem tip cultures of apple, Lane (1978) observed better growth of explants derived from larger buds near branch termini than those of smaller buds near the base. In addition to explant size, bud position along a shoot has influenced explant growth in vitro. Zieslin et al. (1976) reported that growth rate of rose explants increased with

buds located more distally from the shoot tip.

In apple, primary explants such as shoot tips and meristem tips often have been used for in vitro cloning (Appendix Table 1). Other explant sources such as leaf and stem tended to form callus in culture (Skirvin 1981).

2.1.2.3 Somatic embryogenesis

Somatic embryogenesis is a process whereby single somatic cells undergo a series of developmental sequences, including the globular, heart-shape, and torpedo stages, to form bipolar embryoids (Street 1979). Organogenesis refers to the process through which somatic cells develop into unipolar organs such as roots or shoots (Winton 1978). According to Sharp et al. (1980), two patterns of in vitro embryogenesis generally have been recognized: direct and indirect embryogenesis. Direct embryogenesis is characterized by embryoids originating directly from tissues in the absence of callus formation; callus formation is a prerequisite for development of embryoids during indirect embryogenesis. These two patterns of embryogenesis also could be characterized by their relative times of determination and differentiation into embryogenic cells; direct embryogenesis proceeded from preembryogenic-determined cells which were embryogenically determined during prior mitotic divisions; indirect embryogenesis proceeded induced embryogenic determined cells which reentered mitotic cell cycle and redifferentiated into embryo

determined cells (Sharp et al. 1980).

The great potential of somatic embryogenesis for clonal propagation has been recognized (Abbott 1978; Winton 1978). Induction of embryogenesis could be achieved through callus or cell suspension but success has so far been limited mainly to herbaceous plants such as members of the Ranunculaceae, Rutaceae, Solanaceae, and Umbelliferae (Tisserat et al. 1979). A list of plants which have undergone somatic embryogenesis in vitro was compiled by Mott (1979) and Tisserat et al. (1979).

Callus

In apple, callus has been initiated from cotyledons (Chong and Taper 1974a), stems (Chong and Taper 1972), fruits (Lieberman et al. 1979; Pech et al. 1974), anthers (Milewska-Pawliczuk and Kubicki 1977), endosperms (Shih-Kin et al. 1977), and nucellus (Eichholtz et al. 1979a; 1979b), but only one incident of somatic embryogenesis from callus of apple and several woody species have been reported (Lane 1982).

Eichholtz et al. (1979b) reported callus induction from nucellus of Golden Delicious apple fruit 35 to 50 days after anthesis. Embryoids were observed after callus was incubated on hormone-free medium in the dark for 50 days, but plantlet formation was not reported. In Chinese gooseberry (Actinidia chinensis Pl.), embryoids were observed at the periphery of callus derived from stem explants, but plantlet formation was not observed (Harada 1975). Callus grown from unfertilized ovules

(Srinivasan and Mullins 1980) and stem segments (Krul and Worley 1977) of grape was induced to form embryoids from which plantlets were successfully regenerated. Successful plantlet formation from callus culture through somatic embryogenesis also has been demonstrated in two species of papaya, Carica papaya L. (Litz and Conover 1982) and C. candamarcensis Kook. f. (Jordan et al. 1983).

Cell suspension

Somatic embryogenesis has been derived from either free cells or callus masses in suspension (Narayanaswamy 1977). Evidence indicated that embryogenic cells usually originated from cell aggregates (Konar et al. 1972). Although Backs-Husemann and Reinert (1970) demonstrated that the transformation of single isolated carrot cells into embryoids, embryoid formation was later shown to be preceeded by multicellular aggregates (Reinert et al. 1971).

Embryogenesis in vitro was reported in cell cultures of papaya (Litz and Conover 1980; 1983). In C. stipulata Badillo., embryoids were not observed during agitation of cultures for four weeks but only after cultures were left stationary for six to eight weeks (Litz and Conover 1980). Cell suspensions of various woody species have been initiated in apple (Coffin 1974; Lieberman et al. 1979; Pech et al. 1974), sycamore (Acer pseudoplatanus L.) (Street 1977), Douglas fir (Pseudotsuga menziesii (Mirbel) Franco) (Winton 1972), Norway spruce (Picea abies (L.) Karsten) (Winton and Huhtinea 1976), and coffee (Coffea arabica L.) (Monaco et al. 1977), but embryogenesis in these cell suspensions has not been reported.

However, Durzan and Lopushanski (1975) obtained shoots or roots after plating cells from suspension of American elm on agar medium; shoots were rooted in a sterile mixture of sphagnum moss and sand after being treated with 50 mg/L IBA in washed sand for 24 hours.

2.1.3 Factors affecting growth and morphogenesis

Growth and morphogenesis of plant cells and tissues in vitro are governed by the availability of nutrients and prevailing physical environmental factors. A better understanding of these factors can lead to the success of in vitro propagation. Factors affecting plant tissue cultures have been reviewed by Skirvin (1981) for fruit trees, Hughes (1981a) for ornamental species, and Murashige (1974) and Gamborg and Shyluk (1981) for all types of plants.

2.1.3.1 Culture medium

Plant tissue culture media and their components have been reviewed by Huang and Murashige (1977) and Skirvin (1981). The nutritional components usually consist of mineral salts, carbon for energy source, vitamins, and growth regulators. Of all culture media used in plant tissue cultures, the formulation or modifications of MS medium has been used most extensively for both woody and herbaceous species.

Mineral salts

Most media contain the 16 essential elements required for growth and development of plants, although the concentration of each element may vary greatly among media (Huang and Murashige 1977). Tissue culture media generally can be categorized into three types on the basis of the overall salt levels, i.e. high as in MS medium, intermediate as in Cheng's (1978) medium, and low as in White's (1963) medium.

Apple shoots cultured *in vitro* commonly proliferated on medium containing full strength MS mineral salts (Lane 1978; Zimmerman and Broome 1981). Cheng (1978) maintained cultures of apple and other woody plants on medium containing approximately half of MS mineral salt concentrations. Others used White's medium for callus initiation (Letham 1958; Saad and Boone 1964). However, according to Cheema and Sharma (1983), MS medium was superior over other media tested for *in vitro* multiplication and rooting of apple. For rooting of cultured apple shoots, MS mineral salt concentration was reduced by half (Snir and Erez 1980; Sriskandarajah *et al*. 1982), although corresponding full-strength concentration also was successful (James and Thurbon 1981; Jones *et al*. 1979; Jones and Hopgood 1977). According to Hyndman *et al*. (1982a), the beneficial effect on rhizogenesis by lowering total mineral salt level in the rooting medium was probably attributed to increased nitrogen availability to the cultured shoots.

The importance of nitrogen source in the mineral salt fraction has been demonstrated. Nitsch *et al*. (1970) found that ammonium, provided

as ammonium chlorite or ammonium nitrate, promoted growth of apple and pear callus. According to Wetherell and Dougall (1976), induction of embryogenesis required the presence of nitrate and ammonium or amino acids. The concentration of potassium influenced the number of embryoids produced from wild carrot suspension cultures; the number of embryoids produced reached maximum at 10-50 mM potassium; sodium failed to mimic the effect of potassium (Brown et al. 1976). Miller and Murashige (1976) reported that both shoot number and shoot length of several foliage plants were increased with increasing phosphorus concentration. Information with regards to the effect of other major nutrient elements on plant growth and development in vitro is limited (Hughes 1981a; Skirvin 1981).

Of the minor nutrients, iron seems to be the most critical with regards to its influence on growth and development of plant tissue cultures. Occurrence of chlorosis of some fruit tissue cultures was presumably due to iron deficiency (Skirvin 1981). Dalton et al. (1983) found that approximately 45% of iron in MS formulation was lost due to precipitation after two days. However, the addition of extra iron in the medium had little effect on chlorosis of Prunus cultures (Seirlis et al. 1979). Iron is usually added in the chelated form since other forms such as citrate and tartarate are difficult to dissolve and tend to precipitate (Hughes 1981a). Nitsch (1969) demonstrated that chelated iron was more effective in embryoid induction. According to Stultz (1979), chelated iron was not stable in liquid media after autoclaving and might precipitate after a few days. Methods were developed to overcome precipitation during preparation of iron solution (Dalton et al. 1983; Skirvin 1981; Steiner

and Van Winder 1970).

Carbon source

Carbohydrate in the culture medium functions primarily as the source of energy and secondarily as an osmoticum (Thorpe 1980). The most commonly used carbon source is sucrose, although others such as glucose and fructose have been used successfully. Coffin et al. (1976) reported that callus of nine rosaceous species grew better on medium with sucrose; callus of Reliance peach grew better on sorbitol than on sucrose medium. Among 13 carbon sources tested, Chong and Taper (1974a) found that sorbitol, sucrose, glucose, and fructose were the most effective carbon sources for callus growth of apple, although callus of Cortland and Malus robusta Rehd. No. 5 did not grow well on sucrose medium.

The level of sucrose used in most culture media for shoot proliferation and rooting of apple have been 2-3% (w/v) (James and Thurbon 1981; Jones et al. 1977; 1979; Snir and Erez 1980). Zimmerman and Broome (1981) used 1.5% sucrose for root induction of several apple scion cultivars and rootstocks. According to Lane (1978), as the sucrose concentration was reduced to 2%, shoots appeared greener and healthier but root initiation decreased in proportion to the reduction of sucrose; a sharp reduction in rooting occurred with sucrose concentrations higher than 5.2%. Although evidence indicated that sucrose concentrations lower than 3% favored rooting, medium devoid of sucrose often was fatal to cultured shoots. For instance, shoot cuttings of Granny Smith apple died within a week in agitated liquid medium containing no sucrose; 1% sucrose

was the best for root induction (Sriskandarajah and Mullins 1981).

Sucrose concentrations higher than 3% has been used for shoot proliferation and rooting of certain plant tissue cultures. In shoot culture of ornamental plum (Prunus cerasifera Ehrh.), 6% sucrose replaced NAA without affecting shoot proliferation (Zwagerman and Zilis 1979). In Improved Blaze rose, plants produced more and larger roots in media containing 146.1-262.9 mM (50-90 g/L) sucrose than 0-87.6 mM (0-30 g/L) (Hyndman et al. 1982b).

Growth regulator

Early studies of Skoog and Miller (1957) indicated that cytokinin and auxin ratios determined growth and morphogenesis of tobacco cultures in vitro, i.e. high ratios of cytokinin/auxin favored shoot formation, while low ratios favored root formation.

Among cytokinins, BA was most effective in inducing in vitro shoot proliferation of Golden Delicious apple in comparison with kinetin and N^6 (Δ -isopentenyl)-amino purine (2ip) (Lundergan and Janick 1980). Cytokinin alone (Lane 1978; Nemeth 1981; Sriskandarajah et al. 1982) or in combination with auxin (James and Thurbon 1981; Snir and Erez 1980) has commonly been used for shoot proliferation of apple. The greatest shoot proliferation in some rosaceous plants occurred with between 0.1 and 2.5 mg/L BA (Norton and Boe 1982). James and Thurbon (1981) showed that shoot proliferation rate of M.9 apple rotstock from two- to four-fold per month depended on the BA/IBA ratio; a combination of 1-2 mg/L BA and

0.1-0.5 mg/L IBA appeared optimal. Equal amounts of cytokinin and auxin also have been used by other authors (Jones et al. 1977; Snir and Erez 1980). According to Lane (1978), in vitro cultured shoots of McIntosh apple proliferated well only with BA alone; proliferation rate decreased in the presence of NAA.

Rooting of apple shoots has been induced usually by auxin alone (Lane and McDougald 1982; Zimmerman and Broome 1981), although stimulation of rooting by BA and at concentrations of 0.1-0.2 mg/L was reported for several species of Prunus (Nemeth 1979). However, high auxin levels have resulted in excessive callus growth which interfered with rooting (Cheng and Voqui 1977). Lane (1978) indicated that the occurrence of a small amount of callus growth favored rapid induction of root initials in apple.

Jones and Hatfield (1976) found that IBA was better than indoleacetic acid (IAA) and NAA for root induction. In contrast, a relatively high rooting percentage was obtained with NAA by Sriskandarajah et al. (1982) and Lane and McDougald (1982). In addition to IBA, naphthoxyacetic acid also promoted rooting of Granny Smith apple but 2,4-dichlorophenoxylacetic acid (2,4-D) was inhibitory (Sriskandarajah and Mullins 1981). Nemeth tested several uncommon auxin compounds and found that 2-chloro-3-(2,3-dichlorophenyl) propionitrile was even more effective than IBA for root induction of several apple rootstocks. The optimum auxin concentration used for root induction has been in the concentration range of 0.5-3.0 mg/L.

Maximum rooting (100%) of Hally Jolivette cherry was not affected

by the presence or absence of auxin (0.1 mg/L NAA) (Lineberger 1983). Some apple rootstocks such as M.26, M.27, and MM.104 but not Stark Spur apple, were able to form root spontaneously in hormone-free medium (Nemeth 1981). This suggested that differential response to the kind and concentrations of auxin might be due to genotype differences (Nemeth 1981). According to James (1983b), differences in rooting of M.9 and M.26 in response to IAA was attributed to differences in metabolism of endogenous IAA but not differences in its rate of uptake or distribution in the shoots.

In addition to auxin and cytokinin, gibberellic acid (GA) sometimes has been included in shoot proliferating cultures of apple at concentrations of 0.1-0.5 mg/L (Jones et al. 1977; Zimmerman and Broome 1981). For in vitro rooting of Jonagold apple, 1.0 mg/L GA_3 was used to promote root elongation (Druart et al. 1982). The use of other growth regulators such as ethylene and abscisic acid (ABA) for in vitro propagation of apple has not been reported, although they have been used in other plant tissue culture systems (Ammirato 1977; Mele et al. 1982).

Vitamins

Most culture media contain water-soluble vitamins but the kind and concentrations of vitamins depend on the media formulation. According to Hughes (1981a), the addition of vitamins was regarded as a precautionary measure especially when the nutritional requirements for a given plant species for vitamins has not been established. Thiamin was demonstrated to be essential for cell cultures of several plant species (Ohira et al. 1976). While pyridoxin and nicotinic acid promoted growth

of plant cells, other vitamins such as calcium pantothenate, biotin, and ascorbic acid were generally not limiting to growth (Gamborg and Shyluk 1981). On the contrary, Miller et al. (1982) found that riboflavin was completely inhibitory to rooting of Nemaguard peach, and Staba vitamin mixture (Staba 1969) substantially reduced percent rooting; rooting was not affected by the absence or the presence of other vitamins.

Among vitamins, thiamin has always been included in apple tissue culture media. While some media contained only thiamin (Abbott and Whiteley 1976; Jones et al. 1977; Snir and Erez 1980), others included also nicotinic acid, pyridoxin (Lundergan and Janick 1980), calcium pantothenate, ascorbic acid (Nemeth 1981), and biotin (Elliott 1970; Fujii and Nito 1972).

Other organic compounds

A variety of organic compounds, including green tomato extract, coconut milk, orange juice, protein hydrolysate, yeast and malt extracts, often have been added to the culture medium, especially when a defined medium could not be obtained (Skirvin 1981). Some organic compounds such as fruit juice and extract resulted in increasing sugar levels in the medium which could be benefial to growth of cultured cells and tissues in vitro such as citrus (Einset 1978). However, the use of high concentrations of organic compounds could adversely affect cell growth (Gamborg and Shyluk 1981). According to Huang and Murashige (1976), most organic compounds could be replaced by defined nutrients except protein hydrolysate and coconut milk.

Among undefined organic substances, coconut milk is most commonly used in plant tissue culture. Coconut milk contains sorbitol (15,000 ppm in the neutral fraction) (Pollard et al. 1961), cytokinin-like substances, and some reducing nitrogen (Street 1979) but the type and concentrations of most components are unknown. Coconut milk has been used at concentrations of 3 to 5% (Skirvin 1981). Hawker et al. (1973) found that the growth rate of grape berry callus doubled when coconut milk was used instead of casein hydrolysate. In papaya, coconut milk appeared to induce high-frequency embryogenic competency in ovular callus and subsequent embryogeny (Litz and Conover 1982). According to Cutter and Wilson (1974), coconut milk derived from young coconut promoted growth, while that from old coconut inhibited growth of plant tissues.

2.1.3.2 Cultural environment

Effect of cultural environment on growth of plant cell and tissue cultures has been reviewed by Hughes (1981a) and Gamborg and Shyluk (1981). Some of the most important factors are reviewed below.

Temperature

According to Hughes (1981a) temperature influenced metabolism, nutritional needs and morphogenetic events of plant tissues in culture. Most tissue cultures of woody plants have been cultured at temperatures between 23 and 32°C (Skirvin 1981). It was found that temperatures between 28 and 32°C were optimal for growth of apple callus; growth was poor below 20°C (Saad and Boone 1964). Hammerschlag (1982a) observed that during culture initiation, shoot tip survival of Compact Redhaven peach was highest (90%) at 21°C compared to 24, 26, and 28°C. For rooting, temperature was important especially during root initiation of McIntosh apple, with an optimum of 28°C; shoots became chlorotic at lower temperatures (Lane 1978). James (1983a) observed differences in rooting response of M.9 at temperatures of 22, 25, and 29°C. In Myrobalan plum rootstock, percent rooting of shoots cultured at 21 and 26°C was similar but those grown at 26°C developed longer roots than those at 21°C (Hammerschlag 1982b).

Some fruit tissue cultures were demonstrated to be able to tolerate extraordinarily low and high temperatures. For instance, in vitro cultured plantlets of strawberry remained viable after six years of storage at 4°C (Mullin and Schlegel 1976). Shoot tip cultures of Golden Delicious apple survived at 1 or 4°C but not -17°C after storage for up to one year (Lundergan and Janick 1979). Schroeder and Kay (1963) found that avocado (Persea americana Mill.) pericarp tissue survived temperature

as high as 55°C *in vitro* for a few days following pretreatment of tissues at 50°C for 10 min.

Light

Growth and behavior of plant tissues *in vitro* have been greatly influenced by light. The effect of light on plant tissue cultures was mainly attributed to photoperiod, light intensity, and wavelength (Hughes 1981b; Murashige 1974).

Most apple and many other fruit tissue cultures have been cultured under 16-hour photoperiod. Norton and Boe (1982) found that *in vitro* cultured shoots of rosaceous species grown in the dark for a week promoted formation of root initials, except Japanese quince (*Chaenomeles japonica* L.). Rooting of plum shoots was promoted by exposure to dark for two weeks (Hammerschlag 1982b). Grape tissue cultures formed roots only under short days and produced callus under long days (Alleweldt and Radler 1962). Pence *et al.* (1979) observed that rooting of cacao (*Theobroma cacao* L.) was better under 16-hour photoperiod than under continuous dark. Callus of apple (Saad 1965), blackberry (*Prunus serotina* Ehrh.) (Caponetti *et al.* 1971), and blueberry (Nickerson and Hall 1976) have been shown to grow better in dark than light.

Light intensity used for tissue cultures has varied from 1,500 (Eichholtz *et al.* 1979b; Lundergan and Janick 1980) to 6,000 lux (Dutcher and Powell 1972), but most cultures have been maintained between 2,000

and 4,000 lux (Lane 1978; Zimmerman and Broome 1981). Chong and Taper (1974b) found that callus of Cortland apple grew better in the dark, while callus growth of M. robusta was not affected by light intensity between 0 and 7,800 lux; callus growth of McIntosh apple was stimulated by light (7,800 lux). In Robusta coffee, callus grew only in the dark (Startisky 1970).

In tissue culture of Parthenocissus, red light inhibited growth (Butenko 1964). According to Kadkade et al. (1978), red light maximized bud induction of pine cotyledons, while purple and ultra-violet (UV) light had no effect. UV radiation was found to repress growth of ginkgo (Ginkgo biloba L.) pollen culture (Klein 1963).

pH of the medium

The pH of the medium influences availability of nutrients to plant cells and tissues. Growth regulators such as IAA and GA_3, and vitamins were stable at low pH (Butenko 1964), while at high pH, various minor nutrient elements, particularly iron, were unavailable (Street 1957; 1966). The pH of most fruit tissue culture media has been 5.6-5.8 (Skirvin 1981). Tissue cultures of woody plants such as blueberry and rhododendrons grew well at pH of 4.5 (Anderson 1975); peach cells (Sommer and Creasy 1962) and apple callus (Chong 1972) grew well at pH as high as 7.0.

Agar and liquid media

Agar is the most commonly used solidifying agent in tissue culture media, although gelatin, silica gel, and acrylamide gel also have been used (Street 1977). The quality of agar has been a concern since some commercial sources contain small amounts of various undefined organic and inorganic compounds (Dodds and Roberts 1982), including growth inhibitors (Romberger and Tabor 1971). Some authors used liquid media to culture plant tissues to eliminate 'agar effect' (Snir and Erez 1980; Sriskandarajah and Mullins 1981).

Agar concentration varying from 0.5 to 1.0% have been used in fruit tissue culture media (Lundergan and Janick 1980; Zimmerman and Broome 1981), but 0.6-0.8% was preferred (Singha 1982a; Sriskandarajah and Mullins 1981). Singha (1982a) reported that agar concentration higher than 0.3% decreased both shoot proliferation and growth of Almey crabapple, while shoot growth of Seckel pear decreased as agar concentration increased. Romberger and Tabor (1971) also observed a relationship between increase in agar concentration and decrease in growth of spruce meristem in vitro. In apple, rooting was improved as the agar concentration was reduced to 0.27% (Werner and Boe 1980). Growth of peach shoot cultures was significantly greater on liquid than on solid media (Hammerschlag 1982a). In crabapple shoot cultures, Singha (1982a) found decreased growth in terms of fresh weight, dry weight, and number of shoots per explant on liquid media in comparison with solid media. Snir and Erez (1980) obtained higher shoot proliferation of three Malling-Merton apple rootstocks on liquid than on solid medium.

Sriskandarajah and Mullins (1981) reported that percent rooting of apple shoots was much higher in agitated than in stationary liquid media or on solid media. Reduction in root number of M.9 in liquid medium in the presence of phloroglucinol but not on solid medium was observed by James (1983a).

2.2 Sorbitol and related carbohydrates in the Rosaceae

As an active metabolite of major significance, sorbitol (D-glucitol) is a key to the carbohydrate metabolism of apple and other plants in the Rosaceae (Chong and Taper 1974a). In these plants sorbitol was found to be the main product of photosynthesis (Chong and Taper 1971) and a major translocate (Bieleski 1969; Hansen 1970). Sorbitol also functions as intermediate metabolite (Lewis and Smith 1967), respiratory substrate (Fidler and North 1970), and storage compound (Taper and Liu 1969; Whetter and Taper 1966), and was associated with frost hardiness (Pieniazek *et al.* 1978; Sakai 1961) and with certain plant disorders (Braun *et al.* 1971; Rohbrach and Luepschen 1968).

2.2.1 Occurrence and distribution

Sorbitol was first isolated from the fruits of mountain ash (*Sorbus aucuparia* L.), a member of the Rosaceae, by Boussingault in 1872,

and since then was demonstrated to be of general occurrence in other members of this family (Bieleski 1982). According to Plouvier (1955), sorbitol was detected in all members of the subfamilies, Spiridaeoideae, Meloideae, and Prunoideae, but in the subfamily Rosoideae, only the genera Rhodotypos, Kerria, and Neviusia were shown to contain sorbitol. Wallaart (1980) investigated 68 taxa of the Rosaceae, and supported the findings of Plouvier. Wallaart (1980) also found that species with the genome (basic chromosome number) of seven lacked sorbitol, while those with genome of nine accumulated sorbitol. Some authors therefore suggested that the presence or absence of sorbitol could be used for intra-familial classification since distribution of sorbitol in the Rosaceae has been sufficiently clear-cut (Plouvier 1955; 1963; Wallaart 1980).

The amount of sorbitol accumulated in plants varied with the species (Plouvier 1955; Wallaart 1980), organs (Chong 1971; Grant and Rees 1981), time of day (Chong and Taper 1971), or growing season (Chong 1971). In general, tissues such as mature leaves contained 60-80% of soluble carbohydrates as sorbitol, while tissues such as callus, root cuttings and germinating seedlings contained only 15% (Bieleski 1982). Sorbitol was not detected in floral nectars in which glucose, fructose and sucrose were the major carbohydrates (Bieleski and Redgwell 1980), and in seeds during storage (Taper et al. 1972). In mature fruit tissues, sorbitol usually occurred at concentrations of 0.5-2% on a fresh weight basis (Bieleski 1982).

2.2.2 Translocation

Ample evidence indicated that sorbitol was an important translocated carbohydrate in rosaceous plants (Reid and Bieleski 1974; Webb and Burley 1962). According to Zimmermann and Ziegler (1975), the phloem sap normally contained 15-25% sorbitol, 10-20% sucrose and 0.1-2% raffinose and stachyose. In xylem sap the content of sorbitol varied from 0.02 to 0.8% (Hansen and Grauslund 1978; Raese et al. 1978; William and Raese 1974). Bieleski (1982) suggested that the presence of sorbitol in xylem sap might be of importance in terms of distributing energy source for shoot growth in the spring.

2.2.3 Metabolism

The metabolism of sorbitol was closely associated with sugar metabolism but there was lack of evidence as to whether or not sorbitol synthesis occurred by way of the hexose phosphates or by direct reduction of the sugars (Bieleski 1982). It has been suggested that sorbitol synthesis occurred from reduction of glucose (Anderson et al. 1961; 1962; Whetter and Taper 1963). According to Chong and Taper (1971), sorbitol might be synthesized via fructose because of the structural similarity and interconversion between the two carbohydrates. Indirect evidence was

obtained from Bieleski and Redgwell (1977) who found that labelled fructose-6-phosphate (F6P) and fructose were detected much more rapidly than glucose-6-phosphate (G6P) and glucose, after apricot leaves were fed $^{14}CO_2$. Later, Redgwell and Bieleski (1978) isolated sorbitol-6-phosphate (S6P) and sorbitol-1-phosphate (S1P) from plum leaves and suggested that sorbitol was synthesized via the hexose phosphate pathway: F6P⟶S6P⟶sorbitol.

During the past five years, sorbitol enzymes were isolated from various plant tissues, thus further elucidating the nature of sorbitol metabolism. Sorbitol-6-phosphate dehydrogenase (S6PD=aldose-6-phosphate reductase) (Hirai 1979; 1981) was first isolated from loquat leaves and fruits, and later from cotyledons (Yamaki 1980a; 1981), leaves (Loescher et al. 1982; Negm and Loescher 1981), and fruits (Yamaki 1980b) of apple. This enzyme was NADP- and substrate-specific, and catalysed the following reversible reaction (Negm and Loescher 1981):

$$G6P + NADPH + H^+ \rightleftharpoons S6P + NADP^+$$

Another enzyme, sorbitol dehydrogenase (SDH, EC 1.1.1.14) prevalent in animal tissues (Blakeley 1951), was isolated from fruits and cotyledons (Yamaki 1980b), callus tissues (Negm and Loescher 1979), leaves (Loescher et al. 1982) of apple, and tissues of other plants in the Rosaceae (Negm and Loescher 1981). This enzyme was NAD-specific but substrate-non-specific, and catalysed the following reversible reaction (Negm and Loescher 1979):

$$\text{sorbitol} + NAD^+ \rightleftharpoons \text{fructose} + NADH + H^+$$

In addition to S6PD and SDH, sorbitol oxidase (SO) was isolated from leaves and fruits of apple (Yamaki 1980b; 1982a). Sorbitol oxidase was

NAD- and NADP-independent, and was responsible for catalysing the following reaction (Yamaki 1980a):

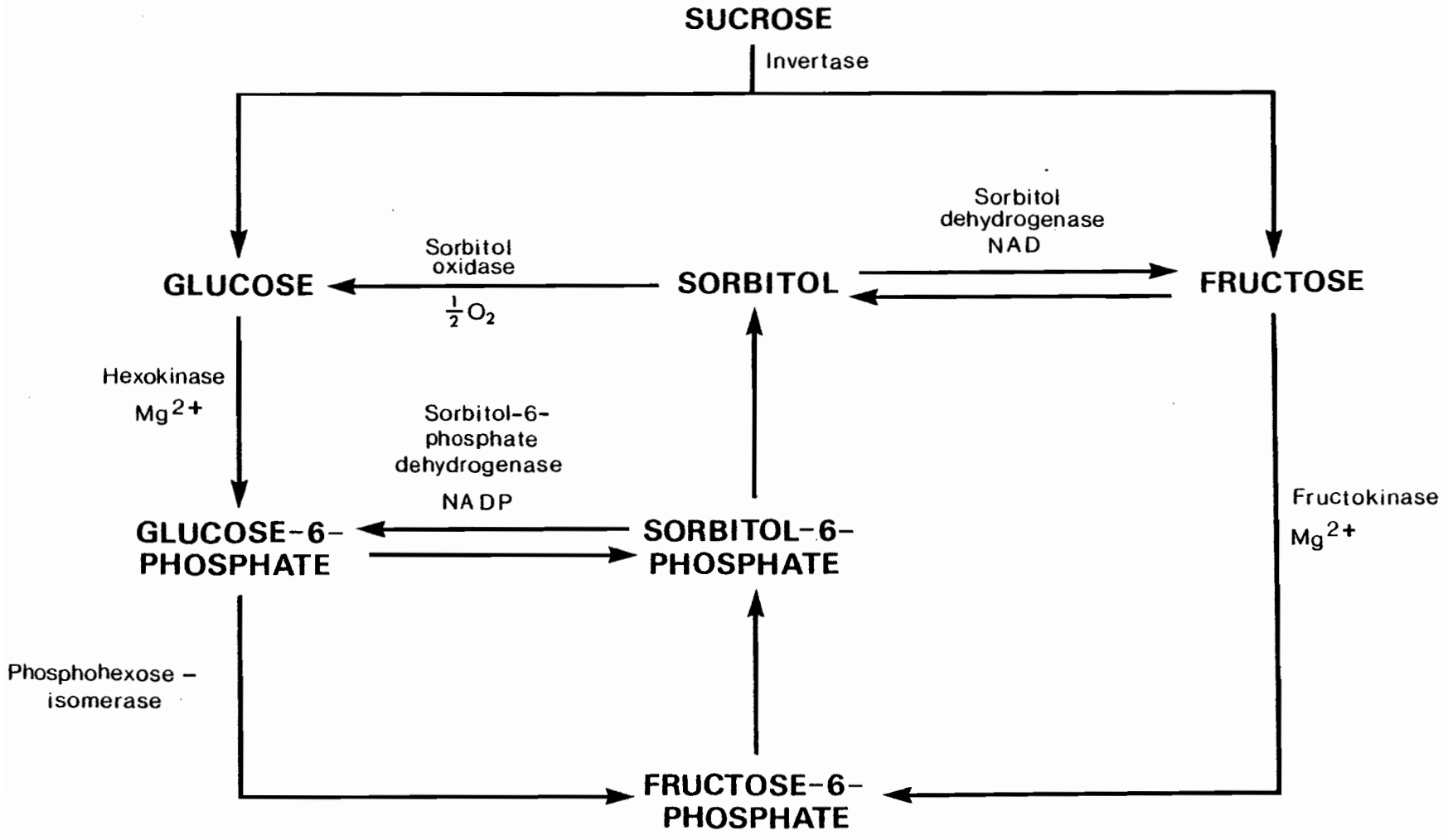
$$\text{sorbitol} + 1/2\ O_2 \longrightarrow \text{glucose} + H_2O$$

The relationship among these enzymes in sorbitol metabolism is illustrated in Figure 2 in a scheme devised by this writer.

Negm and Loescher (1981) found only SDH in immature apple leaves and S6DP in mature leaves, they also detected a decrease in SDH and an increase in S6PD activity, as leaves developing in the spring underwent the transition from sink to source. This suggested that SDH was mainly responsible for degradation and S6PD for synthesis of sorbitol (Negm and Loescher 1981). Yamaki (1981) provided further evidence that S6PD was mainly localized in chloroplasts of mature apple leaves. According to Yamaki (1980b), the highest activity of S6PD and SDH was located in cotyledons and fruit tissues of apple, respectively, while SO showed the weakest activity of the three enzymes in both tissues.

Fruits usually contained little sorbitol after maturation (Chan et al. 1972; Reid and Bieleski 1974), although sorbitol uptake was high during the initial growth phase (Beruter 1983), and 80% sorbitol as total soluble sugars was detected at cell division and pre-enlargement period (Yamaki et al. 1979). Application of radioactive labelled sorbitol to fruits of apple (Hansen 1970) and French prune (Hansen and Ryugo 1979) indicated that sorbitol was readily converted to sucrose, then to glucose and fructose. Yamaki (1980b) reported that the conversion from sorbitol to other sugars in fruit tissues occurred mainly by the enzyme SDH rather

Fig. 2. Proposed pathways of sorbitol metabolism.


SUCROSE
Invertase
Sorbitol dehydrogenase NAD
Sorbitol oxidase
GLUCOSE
SORBITOL
FRUCTOSE
$\frac{1}{2}O_2$
Hexokinase
Mg^{2+}
Sorbitol-6-phosphate dehydrogenase
NADP
Fructokinase
Mg^{2+}
GLUCOSE-6-PHOSPHATE
SORBITOL-6-PHOSPHATE
Phosphohexose – isomerase
FRUCTOSE-6-PHOSPHATE

than SO since the activity of SDH was 11-fold that of SO.

In addition to sorbitol degradation, SO probably also played an important role in sorbitol transport from vacuole to cytoplasm since SO was distributed mainly in the tonoplast membrane, in the interspaces between the cell wall and plasma membrane, and in subcellular organelles (Yamaki 1982a). Carbohydrates in the vacuoles consisted mainly of sorbitol and glucose (Yamaki 1982b). Yamaki (1982a) postulated that SO probably acted as acid invertase in sucrose transport; sorbitol in the vacuole was converted into glucose by SO, then glucose was transported into the cytoplasm where it was converted into sorbitol by S6PD; approximately 50% of the reaction, G6P$\longrightarrow$S6P, catalysed by S6PD occurred in the cytoplasm (Yamaki 1981).

2.3 Juvenility in woody plants

The concept of juvenility has been controversial. Doorenbos (1965) indicated that morphological and physiological characters observed in juvenile plants were strongly correlated to each other. According to Borchert (1976), there was often no high correlation between characters associated with juvenility. He also suggested that each character might have an independent control mechanism. The theoretical and practical aspects of juvenility of woody plants have been reviewed by Borchert (1976), Zimmerman (1972), and Doorenbos (1965).

2.3.1 Phase change

Woody plants normally pass through a series of consecutive growth stages each of which is manifested in a set of different morphological and physiological phenomena during the course of development (Leopold and Krisdemann 1975). Hartmann and Kester (1983) divided these growth stages into embryo, juvenile, transition, and adult phases.

The embryo phase, beginning with the union of male and female gametes, resulted in the formation of a zygote (Hartmann and Kester (1983). According to Zimmerman (1972), the juvenile phase started from seed germination. Juvenile plants were characterized by the distinctive morphology of leaf, stem (thorniness), growth habit, and phyllotaxy; physiologically, these plants showed enhanced vegetative regeneration ability and ability to flower (Doorenbos 1965; Hartmann and Kester 1983). In the transition phase, plants could be recognized by changes in certain morphological characters such as leaf shape of blackwood acacia (Acacia melanoxylon R. Br.) and growth habit of fig (Ficus pumila L.) (Doorenbos 1965), and physiological characters such as reduction in rooting ability (Gardner 1929). Zimmerman (1972) indicated that from a practical viewpoint, the transition phase can be considered as part of the juvenile phase since plants in these two phases are difficult to distinguish from each other. Plants in the adult phase were characterized by increased difficulty in rooting and inability to form flowers (Doorenbos 1965; Zimmerman 1972).

2.3.2 Morphological character

The differences in morphological characters between juvenile and adult plants varied with the species and were not confined to a single character (Doorenbos 1965). In general, the most commonly used morphological characters for growth phase differentiation are growth pattern, leaf shape, phyllotaxy, and the presence of particular structures such as thorns, tendrils, or roots.

2.3.2.1 Growth pattern

The juvenile plants of some woody species such as English ivy (Hedera helix L.) (Stoutemyer and Britt 1969) and fig (Davies et al. 1982) were in the form of vines which crept on the ground, or clung to a support with aerial roots, while corresponding adult plants showed an erect and upright growth pattern. The branching pattern of juvenile plants of M. robusta showed long whip-like branches and a narrower branching angle in comparison with open, smooth branches in adult plants (Blair et al. 1956).

2.3.2.2 Leaf shape and phyllotaxy

The appearance of leaf shape and phyllotaxy were distinctly different between juvenile and adult individuals of some woody plants such as juniper (Juniperus virginianum L.) in which leaves were needle-like in the juvenile and scale-like in the adult phase; leaves of juvenile plants of English ivy were palmate and ovate in the adult plants (Leopold and Krisdemann 1975). Leaves of juvenile English ivy resembled shade leaves of the adult (Bauer and Bauer 1980).

Mullins et al. (1979) showed that the phyllotaxy of juvenile grape plants was spiral and became distichous as the plant matured. While phyllotaxy was opposite in the juvenile and alternate in the adult Eucalyptus, the reverse was true for English ivy (Doorenbos 1965).

2.3.2.3 Stem

The presence of thorns on the stem of some woody plants such as apple, citrus, and locust (Robina spp.) was one of the indications of juvenility. According to Leopold and Krisdemann (1975), the ontogenetic sequences of the tree from juvenility to maturity were exhibited by degree of thorniness ascending from the base to the top of the tree. In grape, unlike adult plants, juvenile plants were characterized by the absence of tendrils (Mullins et al. 1979). Stoutemyer and Britt (1969) showed that

the presence of aerial roots in English ivy was an indicator of juvenility.

2.3.3 Physiological character

Woody plants in the juvenile phase possess various physiological characters which are different from those in the adult phase, but knowledge on the physiological bases of these characters has remained obscure (Borchert 1976). Some of the most important physiological characters are reviewed below.

2.3.3.1 Flowering

Inability to flower has been considered as an important physiological character of plants in the juvenile phase (Doorenbos 1965; Schwabe 1976). Heslop-Harrison and Heslop-Harrison (1970) indicated that inability of juvenile plants to flower might be due to insufficient development of the apical meristem. Evans (1971) also indicated that the achievement of sufficient size in the meristem dome would trigger the commencement of flower development. A rise in synthesis of DNA and RNA and an increase in mitotic activity in the apical meristem was observed when plants passed from the pre-floral to the floral stage (Bronchard and Nougarede 1970).

From a practical point of view, ability to flower indicated the end of the juvenile phase with the sign of the adult stage being the actual production of flowers (Zimmerman 1972). However, some plants flowered only after exposure to certain environmental stimuli such as day length (Leopold and Krisdemann 1975). According to Schwabe (1976), juvenile plants were those that would not flower even when they were exposed to appropriate conditions such as temperature and day length.

2.3.3.2 Rooting

Juvenility was one of the most important factors affecting root initiation (Heuser 1976). This has been demonstrated in Pinus (Libby et al. 1972), Malus (Blair et al. 1956), Quercus (Morgan and McWilliams 1976), and Castanea (Vieitez and Vieitez 1976).

In softwood cuttings of M. robusta, Blair et al. (1956) noted that cuttings derived from juvenile plants rooted more easily than those from adult plants. Quamme and Nelson (1965) detected some root promoting substances in juvenile plants of M. robusta similar to those found in easy-to-root woody plants, and speculated that those substances were probably responsible for ease of rooting in juvenile plants.

Phloridzin, a major phenolic compound of apple tissues (Hutchinson et al. 1959), represented 3-7% of the total leaf dry weight, and also occurred in bark and roots (Williams 1964). One of the degradation

products of phloridzin, phloroglucinol, was found to be beneficial for root induction of apple in the presence of auxin both in vivo (Bassuk et al. 1981) and in vitro (James and Thurbon 1979; Jones and Hopgood 1976). Welander and Huntrieser (1981) reported that phloroglucinol promoted rooting of in vitro cultured shoots of A2 apple rootstock derived from both juvenile and adult phases. However, contradictory results on the beneficial effect of phloroglucinol were also reported (Whiteley and Abbott 1978; Zimmerman and Broome 1981).

According to Paton et al. (1970), reduction in rootability as the plant grew older might be due to the accumulation of a rooting inhibitor. He demonstrated that a rooting inhibitor was present in stem cuttings of all adult Eucalyptus species except E. delupta Bl. which was an easy-to-root species; inhibitor was absent in easy-to-root seedling stems of all Eucalyptus species tested. Using E. grandis Hill ex Maid., Crow et al. (1971) were able to determine the rooting inhibitor as 2,3-dioxabicycle(4,4,0) decane system which was only present in difficult-to-root adult tissues but absent in easy-to-root juvenile tissues.

The differences in rooting ability of cuttings from juvenile and adult plants of M. robusta and Ottawa 3 apple rootstocks might in part be due to anatomical differences of the stem (Nelson 1978). However, differences in stem anatomy of other woody plants such as English ivy (Girouard 1967) and fig (Davies et al. 1982) could not account for the variation of rootability between the juvenile and adult plants in those species.

2.3.3.3 Photosynthesis and translocation

Juvenile and adult plants of English ivy was shown to possess different photosynthetic capacity (Bauer and Bauer 1980). Light saturated net photosynthesis per unit leaf area was about 1.5-fold lower in juvenile than in adult plants. Bauer and Bauer (1980) indicated that higher photosynthetic capacity of the adult plants was mainly due to a higher stomatal and higher residual conductance to the CO_2-transfer in leaves of the plants. Thicker leaf and more chloroplasts per unit leaf area in the adult plant also contributed to higher photosynthetic rate (Haflacher and Bauer 1982).

Clark and Hackett (1980) studied the influence of translocation of photosynthate on phase change by using reciprocal grafting of juvenile and adult plants of English ivy, and found that phase reversion of the adult scion occurred only when the adult scion was defoliated. They suggested that leaves present on the adult scion prevented translocation of the 'juvenile hormone' from the juvenile stock, and that defoliation of the adult scion permited translocation to occur. Doorenbos (1954) also observed phase reversion of the adult partner resulting from defoliation of the adult scion grafted on the juvenile stock of English ivy.

2.3.3.4 Leaf abscission and pigment formation

A plant or plant part in the juvenile phase of some deciduous trees, such as figs and oaks, delayed leaf abscission or retained dead leaves during winter, while in the adult phase leaves usually were shed in autuum (Schaffalitzky de Muckadell 1959). With regards to pigment formation, juvenile leaves of apple and pecan (Carya illinoensis K. Koch) (Doorenbos 1965) produced more anthocyanin than adult leaves. In English ivy, red pigment was present on juvenile stems but absent on adult stems (Stoutemyer and Britt 1969).

2.3.4 Anatomical character

Changes in leaf and stem anatomy accompanied by a phase change in woody plants have received considerable attention. According to Doorenbos (1965), leaves of various forest trees in the juvenile phase were composed of large cells, more intercellular spaces, less palisade and more spongy parenchyma cells than in the adult phase. In Marcgravia ambellata Jacq., leaves of the juvenile plant were characterized by the presence of a papillose epidermis with more stomata in the upper epidermis, less sclerenchymatous idioblasts, and much larger chloroplasts in the mesophyll in comparison with the adult plants (Nillesen and Karstens 1955).

Goodin (1965) found that amount of phloem fiber present in the

stem tissue of English ivy was highest in adult, intermediate in transitional, and least in juvenile plants. Marked differences in the stem anatomy of fig between growth phases were also observed; adult stem tisues contained thicker perivascular sclereids and more cells in the periderm, cortex, phloem, xylem, and pith than juvenile stem tissues (Davies et al. 1982).

2.3.5 Cytological character

Cytological investigations showed that cells of tissue cultures derived from juvenile plants of English and Algerian ivy were generally larger than those of adult plants (Stoutemyer and Britt 1965). Both callus cells derived from juvenile and adult English ivy increased in ploidy level after subcultures were prolonged (Banks-Izen and Polito 1980; Polito and Alliata 1981).

Brinks (1962) suggested that phase changes in woody plants might be related to genetic mechanisms. Schaffner and Nagl (1979) analysed cells of buds and leaves of English ivy and found that nuclei of adult phase cells contained 71% higher DNA content than corresponding juvenile phase cells. Higher leaf cell DNA content in adult than juvenile plants also has been observed by Millikan and Ghosh (1971). However, some authors reported no differences in cell DNA content of leaf, bud, or meristem callus between juvenile and adult plants (Domoney and Timmis 1980; Polito and Alliata 1981).

2.3.6 Biochemical character

Changes in enzyme activities which are responsible for the synthesis and degradation of protein and carbohydrate may be involved in phase changes of woody plants. Ghosh and Millikan (1970) reported that total soluble leaf protein of English ivy decreased quantitatively as the plant grew older. Ascorbic acid oxidase remained unchanged in the juvenile and transitional phases but increased in the adult phase (Millikan and Saniewski 1973). With regards to enzymes in sorbitol metabolism of plants in the Rosaceae, only SDH was found in juvenile leaves, and S6PD in adult leaves (Negm and Loescher 1981).

According to Doorenbos (1965), adult wood of apple contained more starch, reducing sugars, minerals, and nitrogenous compounds but less cellulose and lignin than juvenile wood. However, information regarding the chemical composition in the juvenile and adult phases of woody plants is limited.

2.3.7 Factors affecting juvenility

The most important factors affecting the length of juvenility in woody plants are environment, genotype, and cultural practices. These factors have been reviewed by Doorenbos (1965) and Zimmerman (1972).

2.3.7.1 Environment

Among environmental factors, photoperiod and temperature were of prime importance in affecting the length of juvenility (Zimmerman 1972). Wareing (1961) shortened the juvenile period of birch (Betula spp.) from the usual 5-10 years to less than one year by growing the tree under continuous long photoperiod. Tea crabapple (Malus hupehensis (Pamp.) Rehd.), grown in the greenhouse under continuous long day, flowered approximately 13 months after seeding (Zimmerman 1971). Shortening the juvenile period by growing plants under continuous long photoperiod also has been demonstrated in woody plants such as peach (Lammert 1943), camellia hybrids (Camellia spp.) (Lammerts 1949), azalea-rhododendron hybrids (Rhododendron spp.) (Doorenbos 1955), apple (Karnatz 1970), and pea tree (Caragana sp.) (Heimburger 1958). With the grapefruit (Citrus paradisi Macf.), precocious flowering occurred only with day temperatures at 20-26°C and night temperature at 7°C; day temperatures at 29-38°C prevented flowering even if night temperatures were low (Hield et al. 1966).

2.3.7.2 Genotype

Variation in length of the juvenile phase between plant species is controlled genetically (Johnsson 1949; Teich and Holst 1969; Visser 1965). The length of the juvenile phase of some woody plants has been shortened through selection and breeding. For instance, in birch, Johnsson (1949) obtained progenies with a juvenile phase of only two years compared to normal plants with 5-10 years of juvenile period, through selection of precocious parents. In apple, the trait of short juvenile phase also was transmittable to the progeny (Tydeman and Alson 1965; Visser 1965). According to Zielinski (1963), the transmission of the Cardinal Red leaf gene was resposible for the precocious flowering in pear.

2.3.7.3 Cultural practices

Grafting

Grafting of juvenile plants to mature trees resulted in precocious flowering in apple (Hedrick an Willington 1912), grape (Snyder and Harman 1936), aspen (Populus sp.) (Heimberger 1958), citrus (Furr et al. 1947), and larch (Larix sp.) (Robinson and Wareing 1969). The juvenile period of the scion could be shortened one to three years by grafting onto dwarfing rootstock as demonstrated on apple (Campbell 1961; Tydeman and Alson 1965; Visser 1965; Zimmerman 1972). Some authors failed to shorten the juvenile period of apple and citrus seedlings by grafting the adult scion onto the

seedling rootstocks even though the scions themselves flowered (Curtis 1966; Furr et al. 1947). In English ivy (Doorenbos 1965) and rubber (Hevea brasiliensis Muell.-Arg.) (Muzik and Cruzado 1958), seedling rootstock enhanced juvenile characters of the adult scion.

Pruning

Flowering of fruit trees could be delayed by pruning (Blair et al. 1956; Spinks 1925; Tydeman 1937). For instance, apple seedlings grafted on M.9 and M.12 rootstocks delayed flowering after spurs and leaders were pruned (Tydeman 1937). Delayed flowering by pruning branches also was observed in plums (Crane 1920). In addition, root pruning (Faulkner 1966) or restricted root growth (Spinks 1925) was effective in delaying flowering, although some authors reported contradictory results (Holst 1961; Robinson and Wareing 1969; Zimmerman 1971).

Chemical treatment

The effect of exogenous growth regulators on plant growth and development has been documented (Weaver 1972; Zimmerman 1972). Among growth regulators, GA was most important since juvenile apices contained much higher GA levels than adult apices (Frydman and Wareing 1973a; 1973b). Induction of juvenility by applying GA_3 on the adult plant has been demonstrated in English ivy (Goodin and Stoutemyer 1961; Rogler and Hackett 1975), citrus (Cooper and Peynado 1958), pears (Griggs and Iwakiri 1961), and other deciduous fruit trees (Luckwill 1970). In contrast, GA_3 also was used for precocious flower induction of some fruit trees and

conifers (Pharis et al. 1965; 1969; Pharis and Morf 1967; 1969).

Application of other growth regulators, such as IAA, kinetin, ABA, and (2-chloroethyl)-phosphonic acid (Ethephon) has not been successful in the induction of rejuvenation (Rogler and Hackett 1975).

Tissue culture

Plant tissue culture has increasingly been used as a tool to investigate juvenility of woody plants. Polito and Alliata (1981) reported that growth in terms of the fresh weight growth index of callus derived from meristem of juvenile English ivy plants was approximately 3.5-fold higher than callus growth from adult plants. Juvenile callus was less stable and easier to develop variants, but had greater adaptability to unfavorable media than adult callus (Robbins and Hervey 1970). Interestingly, growth of callus originating from juvenile reversion shoot was less rapid than that from seedlings, but more rapid than that of adult callus (Stoutemyer and Britt 1969). This indicated that juvenile reversion plants and seedlings which have never advanced to adult stage might not be physiologicaliy identical.

Banks (1979) reported that shoots were induced only from callus initiated from juvenile reversion stem of English ivy but not from callus of adult stem. Blueberry shoot tips derived from juvenile plants were generally easier to establish and grew better than those of adult plants in vitro (Lyrene 1978; 1980; 1981).

Rejuvenation is a common phenomenon in plant tissue culture. Mullins *et al.* (1979) found that shoot tips of adult grape plants cultured *in vitro* produced juvenile characters after several subcultures. Lyrene (1981) observed rejuvenation of blueberry in shoot tip cultures and speculated that shoot proliferation through adventitious budding from internodes and leaf margins, and through sprouting of axillary buds might be associated with blueberry rejuvenation. In passion flower (*Passiflora suberosa* L.), callus from the upper part of the plant produced shoots that flowered for several weeks *in vitro*, but lost the ability to flower after being subcultured (Scorza and Janick 1980).

CHAPTER III

IN VITRO PROPAGATION OF OTTAWA 3 APPLE ROOTSTOCK

3.1 Introduction

Ottawa 3, a winter hardy, dwarfing apple (Malus baccata (L.)Borkh. X M. sylvestris Mill. X Jaune de Metz) rootstock (Spangelo et al. 1974), is currently in strong demand but plants are in short supply. Difficulty or inconsistency of propagation by conventional methods has been a major problem with this rootstock (Nelson 1976).

Rapid in vitro multiplication of plantlets from shoot-tip or meristem cultures has been reported for various apple scion cultivars (Jones et al. 1979; Lane 1978) and rootstocks (Nemeth 1981; Werner and Boe 1980). Studies indicate that in vitro apple shoot proliferation is influenced by additions of cytokinins, auxins, and/or GA to a basal

culture medium (Lundergan and Janick 1980), while rooting *in vitro* is influenced more by the presence of auxins alone (Zimmerman and Broome 1981). Strahlheim and Cailloux (1980), studying the influence of the cytokinins, BA, kinetin, and 2ip, obtained shoot meristem cultures of Ottawa 3, most of which were abnormal in growth; when the basal culture medium was supplemented with 0.5 mg/L BA and 40-160 mg/L phloroglucinol, cultures attained a normal appearance but abnormal leaf growth reappeared after two subcultures.

Similar problems in shoot meristem cultures of Ottawa 3, i.e. compact shoots with short internodes and with filiform distorted leaves with margins curling upward or downward (Fig 3A), were encountered initially in both laboratories at Macdonald College and at St-Jean-sur-Richelieu. However, the present study describes methods for obtaining normal shoot cultures (Fig 3B) and for rapid *in vitro* shoot proliferation and rooting of Ottawa 3 apple rootstock from meristem tips.

3.2. Materials and methods

3.2.1 Initiation

In May 1981, bases of 60-70 cm long shoots of Ottawa 3 were immersed in water for two to three days at room temperature to force leaf emergence from buds. Individual buds were removed and scales and outer leaves of each peeled away. Buds were then surface-sterilized by dipping

into 95% ethanol for 5-10 seconds, followed by continuous agitation for five minutes in 10% Javex (Bristol-Myers Canada Inc.) solution (0.6% NaOCl), and rinsing twice with sterile double-distilled water. Meristem tips (ca. 1 mm) were aseptically excised and transferred to 100 x 15 mm petri dishes each with 15 ml of culture medium, described below. Petri dishes were placed in 16 x 14 mm polyethylene bags After four days, contamination-free meristem tips (>95%) were re-transferred to fresh medium and held for four weeks. Cultures were kept in a culture room at 26 ± 2 °C under 16-hour cool white illumination of 51.5 $\mu E\ s^{-1}\ m^{-2}$ (2,500 lux).

The culture medium consisted of MS macro- and micro-elements with iron added as 30 mg/L FeNa EDTA (13% iron; J.T. Baker Chemical Co.) and the following constituents (mg/L): thiourea, 25; asparagine, 180; glycine 2.0; thiamin.HCl, 0.1; pyridoxin.HCl, 0.5; nicotinic acid, 0.5; inositol, 100; sucrose 30,000; Difco-bacto agar, 7,000. In all investigations reported herein, media constituents were added and the pH adjusted to 5.7 before autoclaving at 1 kg/cm^2 and 121 °C for 15 minutes.

3.2.2 Shoot proliferation

Shoots (ca. 5 mm) were subcultured under conditions described above at monthly intervals over a five-month period on similar agar medium supplemented with the growth regulators, BA and NAA, each at 1 mg/L. Shoot growth of these subcultures was abnormal (Fig. 3A). During the sixth subculture, shoots were transferred to experimental media of similar

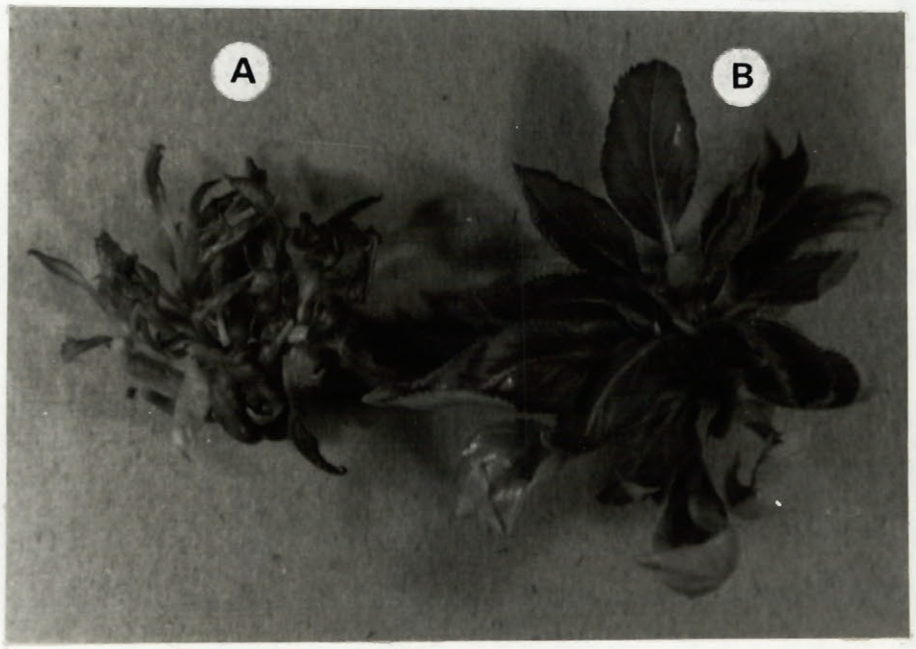


Fig. 3. Abnormal (A) and normal (B) shoot cultures of Ottawa 3 rootstock derived from meristem tips.

composition but containing all combinations of the following growth regulators: BA + NAA (0.1 + 0.1, 0.5 + 0.5, 1.0 + 1.0, and 5.0 + 5.0 mg/L) and GA_3 (0, 0.1, 0.5, 1.0, and 5.0 mg/L). In this investigation, there were four replications and five shoots per growth regulator treatment combination in a 100 x 20 mm petri dish containing 40 ml of culture medium. Shoot proliferation (growth) was evaluated after four weeks in terms of number of shoots, shoot length, and shoot fresh weight. Degree of normality of growth also was evaluated in terms of leaf normality index (LNI) according to the following classification: 0, 1, 2, and 3 as 100%, $>$ 50%, $<$ 50% abnormal leaves, and no abnormal leaves, respectively. Data were analysed as 4 (BA + NAA) x 5 (GA_3) factorial design with four replications.

3.2.3 Rooting

Shoot cuttings (2-3 cm long) from normal cultures (Fig. 3B) were implanted on half-strength (mineral salts) solidified medium supplemented with 50 mg/L inositol, 30,000 mg/L sucrose, and a series of IBA concentrations (0, 0.05, 0.25, 1.25, and 6.25 mg/L). There were five replications and five cuttings per IBA treatment in a 125-ml Erlenmeyer flask containing 40 ml of medium. A preliminary investigation in which shoot cuttings were rooted in half- and full-strength media supplemented with 50 and 100 mg/L inositol, respectively, 30,000 mg/L sucrose, and either 1.0 mg/L IBA or 1.0 mg/L NAA, indicated that rooting (30%) occurred only in the half-strength medium with IBA.

Rooting response was evaluated after two weeks in terms of percent rooting, root number per rooted cutting, and root length per rooted cutting. Data were transformed to $\log_e (x + 0.5)$ for percent rooting, and to $\sqrt{x + 0.5}$ for both root number and root length per rooted cutting, before analysis as a randomized complete block design.

3.3 Results

3.3.1 Shoot proliferation and LNI

Data for shoot proliferation in terms of number of shoots, shoot length, and shoot fresh weight of Ottawa 3 cultured in response to varying combinations of BA + NAA and GA_3 are presented in Table 1. With regards to the main effect of GA_3, there were no significant differences among GA_3 concentrations for all parameters of shoot proliferation except shoot length in which the longest occurred in culture media containing no GA_3 (10.4 mm). Although the main effect of BA + NAA showed that the presence of 0.5 + 0.5 and 1.0 + 1.0 mg/L BA + NAA in the culture medium was most effective for shoot proliferation, increasing GA_3 concentrations resulted in increased shoot proliferation on 0.1 + 0.1 and 0.5 + 0.5 mg/L BA + NAA, but decreased on corresponding 1.0 + 1.0 and 5.0 + 5.0 treatments (Table 1).

Growth was normal when shoots were cultured with 0.5 + 0.5 mg/L

TABLE 1. Influence of concentrations of GA_3 and BA + NAA combinations on shoot proliferation of Ottawa 3 cultures

GA_3 (mg/L)	BA + NAA (mg/L)				Mean
	0.1 + 0.1	0.5 + 0.5	1.0 + 1.0	5.0 + 5.0	
			Number of shoots		
0	1.3[a]	2.9	3.6	1.7	2.4
0.1	1.4	3.1	3.3	1.6	2.4
0.5	1.5	2.8	2.9	1.4	2.2
1.0	1.5	3.2	2.6	1.3	2.2
5.0	1.6	3.5	2.1	1.3	2.1
Mean	1.5	3.1	2.9	1.5	
LSD (P=0.05)		GA_3, NS[b];	BA + NAA, 0.3;	Interaction, 0.7	
			Shoot length (mm)[c]		
0	7.0	11.3	14.3	8.9	10.4
0.1	7.1	11.9	13.3	7.9	10.1
0.5	7.1	10.9	11.2	8.2	9.4
1.0	7.8	11.8	10.9	8.6	9.8
5.0	8.2	12.3	9.9	7.4	9.5
Mean	7.5	11.6	11.9	8.2	
LSD (P=0.05)		GA_3, 0.8;	BA + NAA, 0.7;	Interaction, 1.6	
			Shoot fresh weight (mg)		
0	412	968	1172	758	828
0.1	475	983	1365	703	882
0.5	503	1025	1201	625	839
1.0	515	1166	1034	578	823
5.0	540	1282	905	493	805
Mean	489	1085	1135	631	
LSD (p=0.05)		GA_3, NS;	BA + NAA, 112;	Interaction, 238	

[a] Each datum represents the mean of four replications each with five shoots recorded after four weeks *in vitro*.

[b] Not significantly different.

[c] Length of longest shoot.

BA + NAA in combination with 5.0 mg/L GA_3 (Fig. 3B, 4); with a LNI value of 2.8 (Fig. 4), cultures in this treatment combination exhibited a high frequency of normal leaves and shoots with appreciably longer internodes than those in other treatments. In contrast, all cultures in the presence of high concentrations (5.0 + 5.0 mg/L) of BA + NAA with GA_3 concentrations between 0 and 5.0 mg/L had LNI value of 0 and thus were 100% abnormal (Fig. 4).

3.3.2 Rooting

Data for *in vitro* rooting of Ottawa 3 shoots on half-strength medium are shown in Table 2 . Percent rooting increased progressively with increasing IBA concentrations between 0 and 6.25 mg/L. While minimal callus formation developed at the base of shoot cuttings only in the 6.25 mg/L IBA treatment after seven days, 100% rooting of shoot cuttings was achieved after two weeks in this treatment. This result was confirmed in a subsequent investigation in which shoot cuttings were rooted in IBA treatments of 1.25, 2.50, 3.75, 5.00, and 6.25 mg/L. In these studies, 50% or less rooting occurred in treatments containing 1.25 mg/L or less of IBA.

Plantlets, transferred to pots containing Pro-Mix, acclimatized relatively easily under greenhouse conditions (Fig. 5).

Fig. 4. Influence of concentrations of GA_3 and BA + NAA combinations on leaf normality index of Ottawa 3 shoot cultures *in vitro*.

BA + NAA combinations (mg/L): 0.1 + 0.1 (▲——▲); 0.5 + 0.5 (●——●); 1.0 + 1.0 (○-----○); 5.0 + 5.0 (△-----△).

LSD (p=0.05): GA_3, 0.1; BA + NAA, 0.1; Interaction, 0.2.

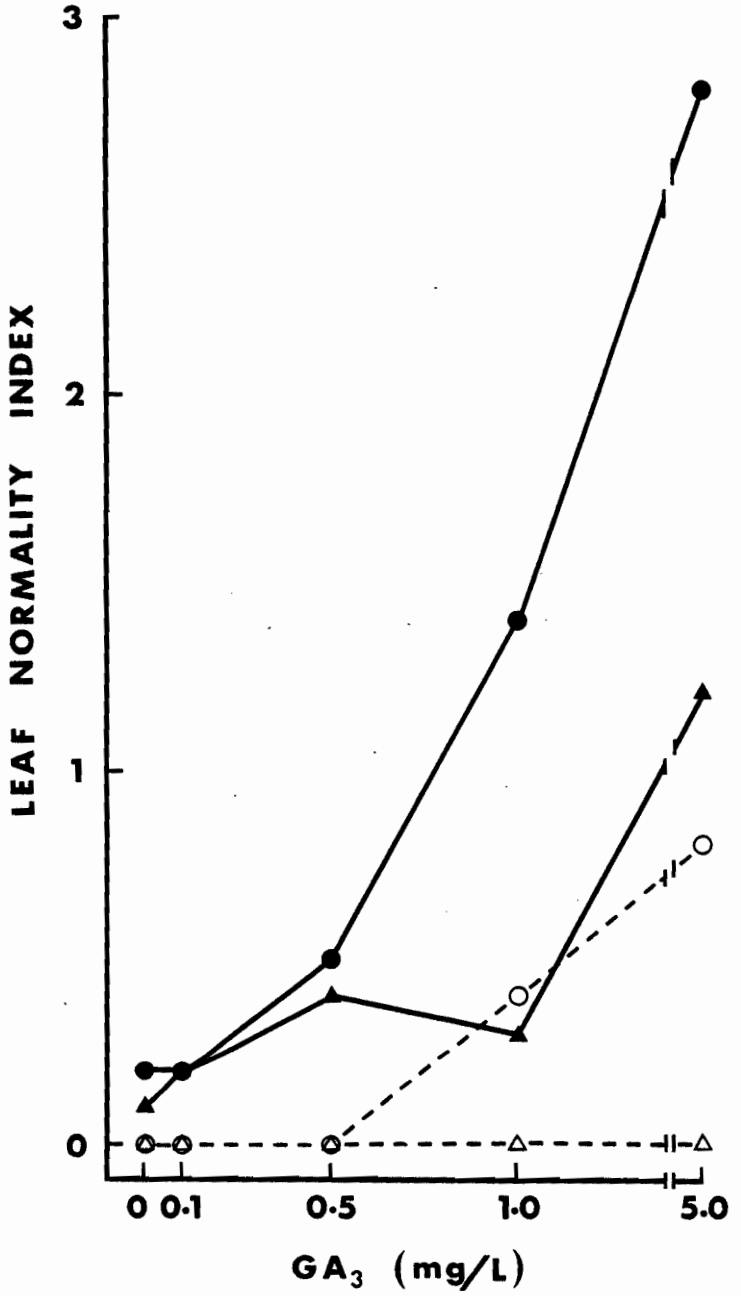

LEAF NORMALITY INDEX
3
2
1
0
0 0·1
0·5
1·0
5.0
GA3 (mg/L)

TABLE 2. Rooting of Ottawa 3 rootstock in response to IBA concentrations after two weeks *in vitro*

IBA (mg/L)	Rooting (%)	Root number per rooted cutting	Root length (mm) per rooted cutting
0	0(-0.69)[a]	0 (0.71)	0 (0.71)
0.05	8(0.79)	0.4(0.91)	2.2(1.64)
0.25	32(3.43)	2.0(1.56)	4.3(2.05)
1.25	44(3.71)	4.3(2.05)	8.8(3.05)
6.25	100(4.61)	4.2(2.17)	7.6(2.85)
LSD (p=0.05)	(1.36)	(1.07)	(1.60)

[a] Each datum represents the mean of four replications each with five shoots cuttings. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

Fig. 5. Acclimatized plantlet of Ottawa 3 rootstock four weeks after being transferred from *in vitro* culture medium.

3.4 Discussion and conclusions

Lundergan and Janick (1980) showed that shoots of Golden Delicious apple cultured *in vitro* with BA at the concentrations most effective for proliferation (3-5 mg/L) were stunted. When normal shoots were achieved by transferring from BA to 2ip or to BA at 1 mg/L with the addition of IBA at 1 mg/L, GA_3 at 0.1 mg/L with BA and/or IBA had no influence on shoot proliferation (Lundergan and Janick 1980). Elliott (1972) reported that apices of Granny Smith apple excised in the spring grew rapidly and formed abnormal, elongated leaves in the presence of 5 or 10 mg/L GA_3 , but with lower concentrations of GA_3 this effect was reduced or absent; apices excised later in the season showed less response to GA_3. Lane (1978) reported an antagonistic effect of *in vitro* proliferation of McIntosh apple seedlings cultured with 1.7 mg/L GA_3 in combination with 0.1 mg/L NAA and 1.1 mg/L BA. In the present study, shoot proliferation of Ottawa 3 (Table 2) with 1.0 + 1.0 or 5.0 + 5.0 mg/L BA + NAA showed increasing inhibition with increasing GA_3 concentrations; at the lower concentrations of BA + NAA (0.1 + 0.1 and 0.5 + 0.5 mg/L) there was either no effect or a tendency towards increasing shoot proliferation with increasing GA_3 concentrations.

This evidence indicates that the effect of GA_3 on *in vitro* apple shoot proliferation can be expected to vary with factors such as interactions with composition and types of other growth regulators or with cultivars or species. Thus, in the case of Ottawa 3 rootstock, the interactive effect of GA_3 with BA + NAA, which restored normality to

cultured shoots (Fig 3, 4), is noteworthy. The occurrence of good growth of apple shoot cultures without auxin has been reported (Abbott and Whiteley 1976; Nemeth 1981).

Uncontrolled contamination of shoot tip (1-2 cm) explants of Ottawa 3, Ottawa 7, M.7, and M.26 rootstocks and of Macspur and Delicious apple cultivars, and slow deterioration from apparent endogenous contamination of established shoot tip cultures of MM.111, Ottawa 3, Ottawa 7, M.7, M.26, and M.27 rootstocks after approximately six months of subculturing, have been major problems in the laboratory. The present cultures of Ottawa 3 derived from meristem tips have been free of such contamination after more than two years of continuous subculturing.

The high frequency of abnormal leaves as reported by Strahlheim and Cailloux (1980) in Ottawa 3 cultures has been corrected under conditions used in the present study. These cultures have remained essentially normal in growth and appearance (Fig. 3B) after more than seven subcultures on agar medium with 5.0 mg/L GA_3 and BA + NAA, both at 0.5 mg/L. While Strahlheim and Cailloux (1980) obtained temporary correction of abnormal growth by using BA with phloroglucinol, the present study reported normal growth by using a relatively high concentration of GA_3 in combination with BA + NAA. Strahlheim and Cailloux (1980) obtained 90% rooting in agar medium with 0.5 mg/L IBA and 81 mg/L phloroglucinol, and 66% rooting in sterilized soilless medium. This study demonstrated that 100% rooting was obtained by using a higher concentration of IBA alone in the rooting medium; rooted shoots transferred to soilless medium in pots acclimatized easily to external conditions (Fig. 5). Chong (1981)

indicated the favorable use of high IBA concentration for stimulating rooting in cuttings of certain difficult-to-root species. The fact that Ottawa 3 is difficult to propagate may explain these special requirements for normal shoot proliferation and optimum rooting *in vitro*.

CHAPTER IV

INFLUENCE OF CARBON SOURCES ON *IN VITRO* PROPAGATION OF *MALUS ROBUSTA* NO. 5 AND OTTAWA 3 APPLE ROOTSTOCKS

4.1 Introduction

Considerable success in using plant tissue culture techniques for rapid clonal propagation of plants has been achieved in recent years primarily through manipulation of the culture media and the environment, and proper selection of explant material. Even so, our ability to regulate organogenesis *in vitro* remains limited especially with regards to woody species (Thorpe 1980). This is largely due to limited knowledge of the physiology and biochemistry of the organ-forming process (Thorpe 1980).

Carbohydrates are of prime importance in organogenesis since organ formation *in vitro* is a high energy-requiring process (Thorpe 1978). A

continuous supply of free sugars from the medium was required for growth of shoot primordia and/or their development into leafy vegetative shoots (Thorpe 1974). Sugar is usually supplied in the medium as sucrose although, in some cases, glucose and other carbohydrates have been equally or more effective than sucrose (Gautheret 1955). In shoot tip and meristem cultures of apple, sucrose has been the standard carbon source used in the nutrient medium for culture initiation and shoot multiplication (Skirvin 1981). However, in an early attempt to initiate meristem tip culture of M. robusta rootstock in this laboratory, explants failed to grow on MS nutrient medium supplied with 30 g/L sucrose. Previous studies by Chong and Taper (1974a) showed that among 13 carbon sources tested, sorbitol, sucrose, glucose, and fructose, each at 3 and 6% (30 and 60 g/L) were most effective for callus growth of McIntosh and Cortland apples and of M. robusta; growth of the Cortland and M. robusta callus cultures were relatively poor on medium supplied with sucrose. For Reliance peach, sorbitol was better than sucrose as carbon source for initiation and growth of callus cultures (Coffin et al. 1976).

Sorbitol is an important carbohydrate constituent in plants of the Rosaceae. In apple, sorbitol functions as the major translocate (Bieleski 1969; Hansen 1970) and metabolite of photosynthesis (Chong and Taper 1971), and is found in almost all plant parts (Chong 1971; Taper and Liu 1969). However, there is virtually no information on organ formation as influenced by sorbitol.

The objective of this study was to compare the influence of sorbitol, sucrose, glucose, and fructose, on initiation of meristem tip

cultures of M. robusta, and on in vitro shoot proliferation and rooting of both M. robusta and Ottawa 3 apple rootstocks.

4.2 Materials and methods

4.2.1 Malus robusta No. 5

4.2.1.1 Initiation

In May 1982, bases of 25-35 cm long shoots of M. robusta collected from both stoolbed and mature tree (ca. 10 years old) sources at Frelighsburg sub-station of Agriculture Canada St.Jean Research Station, Quebec, were immersed in water overnight at room temperature. Using methods described in Section 3.2.1, meristem tips were obtained from individual buds similarly cultured except that thiourea and asparagine were omitted, and 1.0 mg/L IBA was used instead of 1.0 mg/L NAA. After four days, contamination-free explants were transferred to 100 x 20 mm petri dishes with 40 ml of medium supplied with sorbitol, sucrose, glucose, and fructose, each at a concentration of 30 g/L. There were two replicate petri dishes, each with 10 explants per carbon source treatment. Explants were transferred to similar media after four weeks. Fresh weight, leaf number, shoot length, and percent survival of explants were recorded after four and eight weeks.

4.2.1.2 Shoot proliferation

Shoot tips (ca. 5 mm) were subcultured at monthly intervals under conditions described in Section 3.2.1 on sorbitol medium in 100 x 20 mm petri dishes with 40 ml of medium or in 93 x 92 mm ointment jars with 60 ml of medium. For carbon source investigations, shoot tips (stoolbed source) were transferred to similar medium supplied with (a) sorbitol, sucrose, glucose or fructose, each at concentration of 10, 30, 50, and 70 g/L; (b) sorbitol and sucrose combinations (30 g/L total) with varying proportions of each, i.e. sorbitol (%) + sucrose (%): 0 + 100, 25 + 75, 50 + 50, 75 + 25, and 100 + 0. In these investigations, there were four replications and four shoots per treatment in a 125-ml Erlenmeyer flask containing 30 ml of medium. Proliferation in terms of number of shoots, shoot length, and shoot fresh weight was evaluated after four weeks. Since preliminary investigations showed that differential growth of shoots, leaf, and/or stem was influenced by carbon source, leaf/stem ratio (fresh weight basis) was also determined and the compactness of the culture evaluated as follow:

$$\text{Compactness index (CI)} = \frac{H \times W_1 \times W_2}{N} \times \frac{1}{1000}$$

where H is the height, W1 and W2 are the narrowest and the widest width, respectively, and N is the number of shoots. Data were analysed by analysis of variance.

4.2.1.3 Rooting

Shoot cuttings (ca. 2 cm), grown and multiplied on medium with 30 g/L sorbitol, were implanted for rooting on half-strength (mineral salts) agar medium supplemented with 50 mg/L inositol, either 30 g/L sorbitol or sucrose, and a series of IBA concentrations (0, 0.1, 0.5, and 1.0 mg/L). There were four replications and five cuttings per treatment in 125-ml Erlenmeyer flasks containing 40 ml of medium. Rooting response was evaluated after 32 days and data were transformed as described in Section 3.2.3 before analysis as a factorial experiment.

4.2.2 Ottawa 3

4.2.2.1 Shoot proliferation

Cultures derived from Section 3.2.2 were used in this study. Explants were proliferated on similar sucrose medium as described previously for this rootstock (Section 3.2.1) except that thiourea and asparagine were omitted and the medium was supplemented with 0.5 mg/L IBA, 0.5 mg/L BA, and 3.0 mg/L GA_3 (instead of 0.5 mg/L NAA, 0.5 mg/L BA, and 5.0 mg/L GA_3) since this modified medium seemed to result in slight improvement in culture growth. Investigation of carbon source effect was conducted by transferring shoot tips to medium similar to that used for shoot proliferation but supplied with different carbon sources or

combinations (30 g/L total) of sorbitol and sucrose as described for M. robusta. Shoot proliferation was evaluated as previously described for M. robusta. LNI was also evaluated according to the method described in Section 3.2.2.

4.2.2.2 Rooting

Shoot cuttings (2-3 cm) were implanted on similar rooting medium as used for M. robusta but supplemented with 6.25 mg/L IBA (Section 4.2.1.3) and with different carbon sources, sorbitol, sucrose, glucose, and fructose, each at 10, 30, 50, and 70 g/L. There were four replications and five cuttings per treatment in a flask containing 40 ml of medium. After two weeks, rooting response was evaluated and data were transformed as described in Section 3.2.3 before analysed as a factorial experiment.

4.3 Results

4.3.1 *Malus robusta* No. 5

4.3.1.1 Initiation

Meristem tip explants of *M. robusta* grew during the first four weeks on media supplied with sorbitol, sucrose, glucose, and fructose as presented in Table 3. However, while all explants on sucrose, glucose, and fructose media deteriorated and died after eight weeks, 90% and 50% of explants from stoolbed and mature tree sources, respectively, survived on sorbitol medium (Table 3). Unfortunately, proliferating cultures from the mature tree source were lost due to contamination after three subcultures on sorbitol medium. A proliferation rate of 6- to 10-fold (stoolbed source) was maintained on sorbitol medium (Fig. 6). Cultures on this medium were compact with relatively short shoots, and red pigmentation was usually present on stems, petioles, and leaves, particularly those in contact with the medium.

4.3.1.2 Shoot proliferation

Data for shoot proliferation of *M. robusta* shoot cultures are presented in Table 4. On media supplied with sorbitol, sucrose, glucose, and fructose, the most effective carbon source for shoot proliferation

TABLE 3. Influence of carbon sources on *in vitro* initiation and survival of *Malus robusta* No. 5 meristem tip explants

Carbon source (30 g/L)	After four weeks: Fresh weight (mg) per explant	Leaf number per explant	Shoot length (mm) per explant	Survival (%)	After eight weeks: Survival (%)
			Stoolbed source		
Sorbitol	72 ± 3[a]	4.4 ± 0.4	9.7 ± 1.0	100 ± 0	90 ± 10
Sucrose	24 ± 2	2.3 ± 0.3	6.2 ± 0.7	100 ± 0	0 ± 0
Glucose	24 ± 4	2.2 ± 0.3	6.3 ± 0.7	100 ± 0	0 ± 0
Fructose	35 ± 4	2.4 ± 0.3	6.7 ± 0.9	100 ± 0	0 ± 0
			Mature tree source[b]		
Sorbitol	37 ± 4	2.4 ± 0.3	7.9 ± 0.8	100 ± 0	50 ± 20
Sucrose	14 ± 2	1.6 ± 0.2	5.4 ± 0.5	100 ± 0	0 ± 0
Glucose	24 ± 4	1.9 ± 0.2	6.5 ± 0.6	70 ± 10	0 ± 0
Fructose	20 ± 4	1.5 ± 0.2	4.8 ± 0.4	50 ± 10	0 ± 0

[a] Each datum represents the mean ± S.E. of two replicate petri dishes each with 10 meristem tip explants.

[b] Fruiting trees approximately 10 years old.

Fig. 6. Proliferating shoot cultures of Malus robusta No. 5 from stoolbed source grown on nutrient medium supplied with 30 g/L sorbitol.

TABLE 4. Influence of carbon sources and their concentrations on shoot proliferation of Malus robusta No.5 cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Number of shoots			
Sorbitol	1.9[a]	9.5	4.6	3.0	4.8
Sucrose	1.2	2.5	4.4	2.1	2.6
Glucose	1.4	6.4	3.6	3.3	3.7
Fructose	1.0	1.0	1.0	1.4	1.1
Mean	1.4	4.9	3.4	2.4	
LSD (p=0.05)	Carbon source, 0.9; Concn., 0.9; Interaction, 1.8				
		Shoot length (mm)[b]			
Sorbitol	10.6	16.2	10.7	8.3	11.5
Sucrose	10.1	11.4	10.8	8.6	10.2
Glucose	9.5	14.9	10.6	7.7	10.7
Fructose	7.3	8.7	7.2	7.1	7.6
Mean	9.4	12.8	9.8	7.9	
LSD (p=0.05)	Carbon source, 1.6; Concn., 1.6; Interaction, NS[c]				
		Shoot fresh weight (mg)			
Sorbitol	232	354	227	119	233
Sucrose	219	189	182	101	173
Glucose	221	301	180	167	217
Fructose	110	196	179	171	164
Mean	196	260	192	130	
LSD (p=0.05)	Carbon source, 31; Concn., 31; Interaction, 62				

[a] Each datum represents the mean of four replications each with four shoots recorded after four weeks in vitro.
[b] Length of longest shoot.
[c] Not significantly different.

was sorbitol. With regards to the main effect of carbon source, the highest number of shoots occurred on sorbitol medium (4.8); corresponding values of this parameter on the other carbon source media were in the descending order glucose (3.7), sucrose (2.6), and fructose (1.1) (Table 4). Although the main effect of concentration showed that the highest number of shoots occurred at a carbon source concentration of 30 g/L, the concentration of each carbon source influencing number of shoots was variable. The highest number of shoots occurred at a concentration of 30 g/L for both sorbitol and glucose, 50 g/L for sucrose, and no differences among concentrations for fructose (Table 4).

The significant main effects of carbon source and concentration showed that cultures grown on fructose medium (7.6 mm) had the shortest shoot length, while those grown on sorbitol (11.5 mm), sucrose (10.2 mm), and glucose (10.7 mm) were not significantly different. The tallest shoot length occurred at a concentration of 30 g/L for all carbon sources (Table 4). With regards to shoot fresh weight, shoots grown on sorbitol (233 mg) and glucose (217 mg) were significantly higher than those on sucrose (173 mg) and fructose (164 mg). Similar to number of shoots, the optimum concentration for shoot fresh weight varied with carbon sources: 30 g/L for both sorbitol and glucose; 10 g/L for sucrose; 30 to 70 g/L for fructose (Table 4). On fructose medium, shoots were notably distorted, with a translucent, water-soaked appearance, and callus developed at the shoot base and lower leaves in contact with the medium.

Cultures exhibited marked differences in leaf and stem growth on different carbon source media. A significant main effect of carbon source showed that the lowest leaf fresh weight occurred on fructose medium (113 mg), while those on other carbon source media (129-154 mg) were not significantly different (Table 5). Stem fresh weight on the various carbon source media were in the descending order sorbitol (98 mg), glucose (63 mg), fructose (49 mg), and sucrose (45 mg). The highest leaf and stem fresh weight occurred at a concentration of 30 g/L, and at 30 and 50 g/L for both sucrose and fructose.

Significant differences for leaf/stem ratio and CI of cultures grown on different carbon source media are shown in Table 6. With regards to the main effect of carbon source, the highest leaf/stem ratio occurred on sucrose (3.2) and glucose (3.3) media, intermediate on fructose medium (2.3), and least on sorbitol medium (1.5). A significant concentration effect showed that the highest leaf/stem ratio occurred at 10 g/L for all carbon sources. On the other hand, cultures grown on sorbitol medium were more compact with the lowest CI (1.3); the CI for the other carbon sources were in the ascending order glucose (1.7), sucrose (1.9), and fructose (2.4) (Table 6). The concentration influencing CI varied with carbon sources; the lowest CI occurred at concentrations between 30 and 70 g/L for sorbitol, 50 g/L for sucrose, 30 g/L for glucose, and 10 g/L for fructose.

The response of M. robusta shoot cultures to media supplied with different sorbitol and sucrose combinations (30 g/L total) is shown in Table 7. Increasing the proportion of sorbitol and decreasing that of

TABLE 5. Influence of carbon sources and their concentrations on leaf and stem fresh weight of Malus robusta No. 5 shoot cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Leaf fresh weight (mg)			
Sorbitol	154[a]	195	126	66	135
Sucrose	171	146	124	74	129
Glucose	183	206	120	106	154
Fructose	76	133	125	117	113
Mean	146	170	124	91	
LSD (p=0.05)	Carbon source, 25; Concn., 25; Interaction, 49				
		Stem fresh weight (mg)			
Sorbitol	78	159	101	52	98
Sucrose	48	46	58	27	45
Glucose	38	96	54	62	63
Fructose	34	55	54	54	49
Mean	50	89	67	49	
LSD (p=0.05)	Carbon source, 12; Concn., 12; Interaction, 24				

[a] Each datum represents the mean of four replications each with four shoots recorded after four weeks in vitro.

TABLE 6. Influence of carbon sources and their concentrations on leaf/stem ratio and CI of Malus robusta No. 5 shoot cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Leaf/stem ratio			
Sorbitol	2.1[a]	1.3	1.4	1.4	1.5
Sucrose	3.9	3.5	2.2	3.1	3.2
Glucose	4.9	2.3	2.2	2.4	3.3
Fructose	2.3	2.4	2.3	2.2	2.3
Mean	3.3	2.4	2.0	2.3	
LSD (p=0.05)	Carbon source, 0.7; Concn., 0.7; Interaction, NS[b]				
		CI[c]			
Sorbitol	2.1	0.8	1.1	1.1	1.3
Sucrose	2.9	1.7	1.1	1.7	1.9
Glucose	2.8	1.0	1.5	1.5	1.7
Fructose	1.9	2.7	2.3	2.8	2.4
Mean	2.4	1.6	1.5	1.8	
LSD (p=0.05)	Carbon source, 0.4; Concn., 0.4; Interaction, 0.8				

[a]Each datum represents the mean of four replications each with four shoots recorded after four weeks in vitro.

[b]Not significantly different.

[c]Compactness index.

TABLE 7. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on proliferation of Malus robusta No. 5 shoot cultures

So + Su[a]	Number of shoots	Shoot[b] length (mm)	Shoot fresh weight (mg)	Leaf fresh weight (mg)	Stem fresh weight (mg)	Leaf/stem ratio	CI[c]
0 + 100	2.5[d]	11.4	189	147	43	3.5	1.7
25 + 75	5.4	13.1	233	154	79	2.2	1.1
50 + 50	5.3	13.9	240	166	74	2.4	1.3
75 + 25	6.4	13.3	289	163	125	1.3	0.8
100 + 0	9.2	16.2	354	195	159	1.3	0.8
LSD (p=0.05)	2.4	NS[e]	57	NS	32	0.9	0.5

[a]Sorbitol (%) + sucrose (%).

[b]Length of longest shoot.

[c]Compactness index.

[d]Each datum represents the mean of four replications each with four shoots recorded after four weeks in vitro.

[e]Not significantly different.

sucrose in the carbon source fraction resulted in significantly increased growth compactness of cultures, and higher number of shoots and shoot fresh weight. Accompanying decreases in both leaf/stem ratio and CI were attributed to a decrease in stem fresh weight (Table 7).

4.3.1.3 Rooting

Maximum rooting (85%) of M. robusta shoots (stoolbed source) occurred after 32 days on half-strength medium with 0.5 mg/L IBA in the presence of either sorbitol or sucrose (Table 8). Unlike percent rooting and root length per rooted cutting, root number per rooted cutting was significantly higher on sucrose than on sorbitol medium.

Interestingly, root initiation and development on sorbitol medium were approximately four days slower than those on sucrose medium during the first 28-day rooting period, but there were no apparent differences after 32 days (Fig. 7). Those results confirm other investigations in which IBA was supplied at concentrations between 0 and 5 mg/L.

The in vitro rooted shoots (Fig. 8A) of M. robusta acclimatized relatively easily in pots containing Pro-Mix under greenhouse conditions (Fig. 8B).

TABLE 8. In vitro rooting of Malus robusta No. 5 rootstock in response to IBA concentrations in the presence of sorbitol or sucrose

Carbon source (30 g/L)	IBA (mg/L) 0	0.1	0.5	1.0	Mean
			Rooting (%)		
Sorbitol	0(-0.69)[a]	35(3.53)	85(4.45)	40(3.63)	40(2.73)
Sucrose	0(-0.69)	30(3.36)	85(4.45)	50(3.80)	41(2.73)
Mean	0(-0.69)	33(3.45)	85(4.45)	45(3.72)	
LSD (p=0.05) IBA, (0.15); Carbon source, (NS[b]); Interaction, (NS)					
			Root number per rooted cutting		
Sorbitol	0(0.71)	1.5(1.41)	2.0(1.58)	2.6(1.76)	1.5(1.36)
Sucrose	0(0.71)	1.9(1.55)	2.5(1.73)	3.3(1.95)	1.9(1.45)
Mean	0(0.71)	1.7(1.48)	2.3(1.66)	3.0(1.86)	
LSD (p=0.05) IBA, (0.06); Carbon source, (0.15); Interaction, (NS)					
			Root length (mm) per rooted cutting		
Sorbitol	0(0.71)	17(4.22)	13(3.61)	8(2.97)	10(2.88)
Sucrose	0(0.71)	19(4.42)	13(3.71)	7(2.70)	10(2.89)
Mean	0(0.71)	18(4.32)	13(3.66)	8(2.84)	
LSD (p=0.05) IBA, (0.15); Carbon source, (NS); Interaction, (NS)					

[a]Each datum represents the mean of four replications each with five shoot cuttings recorded after 32 days in vitro. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

[b]Not significantly different.

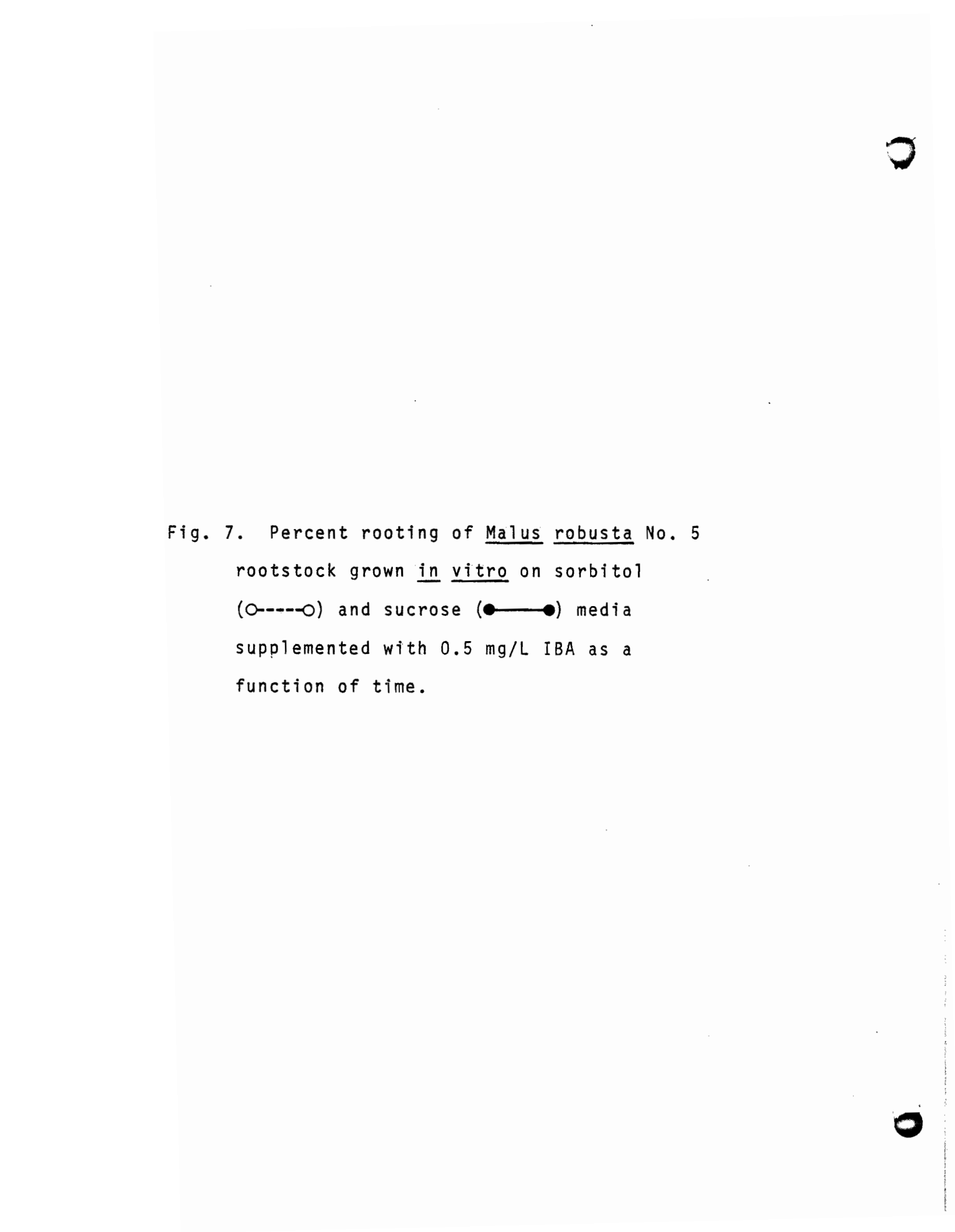


Fig. 7. Percent rooting of *Malus robusta* No. 5 rootstock grown *in vitro* on sorbitol (○-----○) and sucrose (●——●) media supplemented with 0.5 mg/L IBA as a function of time.

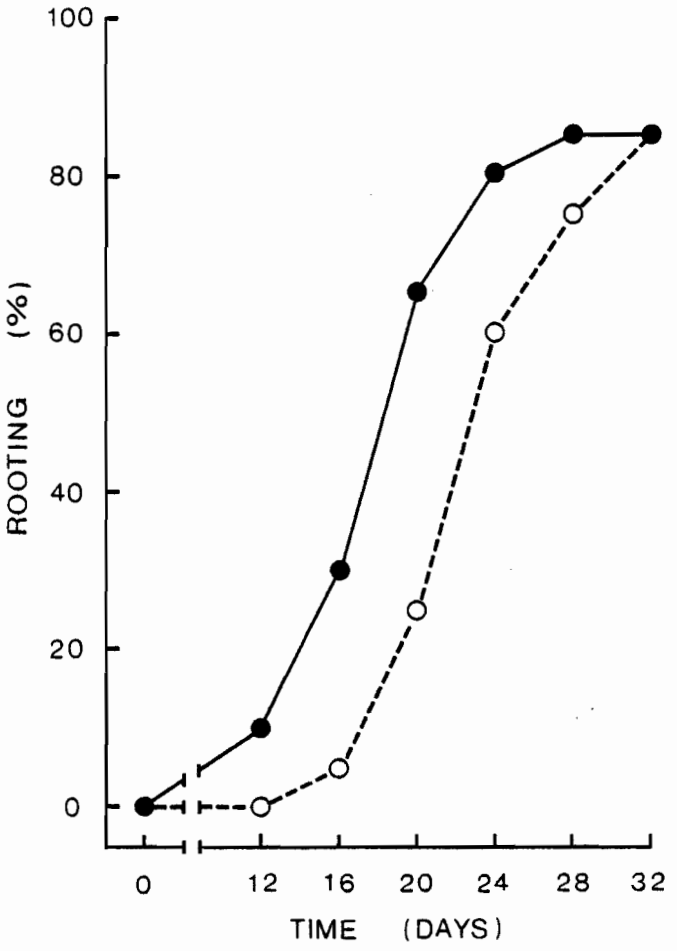

100
80
60
40
20
0
ROOTING (%)
0
12
16
20
24
28
32
TIME (DAYS)

Fig. 8. In vitro rooted shoot (A) and acclimatized plant (B) of Malus robusta No. 5 rootstock.

4.3.2 Ottawa 3

4.3.2.1 Shoot proliferation

Data for shoot proliferation of Ottawa 3 cultures on media supplied with different carbon sources are presented in Table 9. With regards to carbon source effect, cultures grown on fructose medium (2.0) exhibited the least number of shoots, and shortest shoot length on sorbitol medium (11.8 mm); those on other carbon source media exhibited similar number of shoots (2.5–2.8) and shoot length (13.9–14.3 mm) (Table 9). A significant concentration effect showed that the highest number of shoots occurred at 30 and 50 g/L for all carbon sources, while the tallest shoot length occurred at 30 g/L on sorbitol, glucose, and fructose media, and at 30 and 50 g/L on sucrose medium. With regards to shoot fresh weight, there were no differences among carbon sources; the highest shoot fresh weight occurred at a concentration of 30 g/L (Table 9).

Unlike M. robusta (Table 5), leaf and stem fresh weight of Ottawa 3 cultures showed similar response to different carbon source media (Table 10). Leaf fresh weight was highest at carbon source concentration of 30 g/L, and stem fresh weight at 30 and 50 g/L. Significant main effects of carbon source and concentration showed that leaf/stem ratio on sucrose (4.7) and glucose (3.9) media was significantly higher than that on sorbitol (3.3) and fructose (3.3) media, while leaf/stem ratio decreased with increasing carbon source concentrations (Table 10).

TABLE 9. Influence of carbon sources and their concentrations on shoot proliferation of Ottawa 3 cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Number of shoots			
Sorbitol	1.5[a]	3.8	3.7	2.2	2.8
Sucrose	1.5	4.1	3.5	1.8	2.7
Glucose	1.2	3.4	3.4	1.8	2.5
Fructose	1.3	3.2	2.1	1.5	2.0
Mean	1.5	3.6	3.2	1.8	
LSD (p=0.05)	Carbon source, 0.5; Concn., 0.5; Interaction, NS[b]				
		Shoot length (mm)[c]			
Sorbitol	11.9	15.6	11.9	7.7	11.8
Sucrose	10.4	18.5	18.8	9.0	14.2
Glucose	11.2	20.4	15.8	9.8	14.3
Fructose	11.4	20.9	14.7	8.7	13.9
Mean	11.2	18.5	15.1	9.0	
LSD (p=0.05)	Carbon source, 1.5; Concn., 1.5; Interaction, 3.1				
		Shoot fresh weight (mg)			
Sorbitol	470	673	509	338	498
Sucrose	577	704	552	328	540
Glucose	571	794	522	189	519
Fructose	559	814	481	234	522
Mean	544	746	516	272	
LSD (p=0.05)	Carbon source, NS; Concn., 89; Interaction, NS				

[a]Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.
[b]Not significantly different.
[c]Length of longest shoot.

TABLE 10. Influence of carbon sources and their concentrations on leaf and stem fresh weight, and leaf/stem ratio of Ottawa 3 shoot cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Leaf fresh weight (mg)			
Sorbitol	393[a]	492	322	231	360
Sucrose	525	560	397	203	423
Glucose	510	617	360	113	400
Fructose	480	582	325	163	388
Mean	477	563	351	179	
LSD (p=0.05)	Carbon source, NS[b]; Concn., 71; Interaction, NS				
		Stem fresh weight (mg)			
Sorbitol	77	181	187	107	138
Sucrose	52	144	155	119	118
Glucose	61	177	162	76	119
Fructose	79	232	256	71	135
Mean	67	184	165	93	
LSD (p=0.05)	Carbon source, NS; Concn., 20; Interaction, NS				
		Leaf/stem ratio			
Sorbitol	5.1	2.8	1.8	2.2	3.3
Sucrose	10.6	3.9	2.6	1.8	4.7
Glucose	8.5	3.6	2.1	1.5	3.9
Fructose	6.0	2.6	2.2	2.3	3.3
Mean	7.6	3.2	2.2	2.0	
LSD (p=0.05)	Carbon source, 0.8; Concn., 0.8; Interaction, NS				

[a]Each datum represents the mean of four replications each with five shoots recorded after four weeks *in vitro*.
[b]Not significantly different.

With regards to carbon source effect for both LNI and CI, sucrose was the highest, glucose and fructose were intermediate, and sorbitol was the lowest (Table 11). A significant concentration effect showed that the highest LNI occurred at a concentration of 30 g/L, while corresponding CI decreased with increasing carbon source concentrations.

On media supplied with different sorbitol and sucrose combinations (30 g/L total), only shoot length, LNI, and CI showed significant differences, i.e. a decrease with increasing proportion of sorbitol and decreasing proportion of sucrose in the carbon source fraction (Table 12).

4.3.2.2 Rooting

Rooting response in terms of percent rooting, root number per rooted cutting, and root length per rooted cutting of Ottawa 3 shoot cultures on different carbon source media is presented in Table 13. With regards to a main effect of carbon source for percent rooting, sucrose (79%) was most effective, fructose (35%) and glucose (25%) were intermediate, and sorbitol (4%) was least effective. Similar to percent rooting, cuttings on different carbon source media showed significant differences in root number per rooted cutting; the highest root number per rooted cutting occurred on sucrose medium (3.0), intermediate on fructose (1.9) and glucose (1.3) media, and least on sorbitol medium (0.4). Although the main effect of concentration showed that the highest percent rooting and root number per rooted cutting occurred at carbon

TABLE 11. Influence of carbon sources and their concentrations on LNI and CI of Ottawa 3 shoot cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
			LNI[a]		
Sorbitol	0.1[b]	1.4	1.3	0.5	0.8
Sucrose	0.5	3.0	2.5	1.0	1.8
Glucose	0	2.4	1.5	1.1	1.3
Fructose	0.3	2.0	1.5	0.6	1.1
Mean	0.2	2.2	1.7	0.8	
LSD (p=0.05)	Carbon source, 0.4;		Concn., 0.4;	Interaction, NS[c]	
			CI[d]		
Sorbitol	11.5	4.3	2.9	2.5	5.3
Sucrose	9.2	9.7	6.6	4.8	7.6
Glucose	10.5	6.3	4.5	4.3	6.4
Fructose	9.4	8.5	4.9	3.1	6.5
Mean	10.2	7.2	4.7	3.7	
LSD (p=0.05)	Carbon source,	0.9;	Concn., 0.9;	Interaction, NS	

[a] Leaf normality index.

[b] Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[c] Not significantly different.

[d] Compactness index.

TABLE 12. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on proliferation of Ottawa 3 shoot cultures

So + Su[a]	Number of shoots	Shoot[b] length (mm)	Shoot fresh weight (mg)	Leaf fresh weight (mg)	Stem fresh weight (mg)	Leaf/stem ratio	LNI[c]	CI[d]
0 + 100	3.2[e]	17.9	699	588	114	5.3	3.0	10.4
25 + 75	3.0	18.9	779	641	138	4.7	1.5	8.8
50 + 50	3.5	17.0	837	680	137	4.9	1.1	6.7
75 + 25	4.3	15.1	846	694	132	5.2	1.0	5.7
100 + 0	3.1	13.7	666	542	124	4.5	1.0	4.5
LSD (p=0.05)	NS[f]	1.4	NS	NS	NS	NS	0.9	0.8

[a] Sorbitol (%) + sucrose (%).

[b] Length of longest shoot

[c] Leaf normality index.

[d] Compactness index.

[e] Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[f] Not significantly different.

TABLE 13. Influence of carbon sources and their concentrations on *in vitro* rooting of Ottawa 3 rootstock

Carbon source	Concn. (g/L) 10	30	50	70	Mean
			Rooting (%)		
Sorbitol	5(0.24)[a]	5(0.24)	5(0.24)	0(-0.69)	4(0.05)
Sucrose	80(4.38)	100(4.61)	90(4.49)	45(3.70)	79(4.29)
Glucose	25(2.43)	40(3.53)	25(3.19)	10(0.41)	25(2.39)
Fructose	0(-0.69)	60(4.08)	50(3.80)	30(2.54)	35(2.43)
Mean	28(1.59)	51(3.12)	43(2.93)	21(1.49)	
LSD (p=0.05) Carbon source, (0.98); Concn., (0.98); Interaction, (1.96)					
			Root number per rooted cutting		
Sorbitol	0.8(1.00)	0.5(0.93)	0.3(0.84)	0(0.71)	0.4(0.84)
Sucrose	3.0(1.87)	3.8(2.06)	3.2(1.92)	2.1(1.62)	3.0(1.63)
Glucose	1.3(1.27)	2.3(1.63)	1.4(1.35)	0.3(0.84)	1.3(1.25)
Fructose	0(0.71)	3.0(1.85)	2.3(1.88)	2.2(1.55)	1.9(1.09)
Mean	1.3(1.21)	2.4(1.62)	1.8(1.50)	1.2(1.18)	
LSD (p=0.05) Carbon source, (0.23); Concn., (0.23), Interaction, (0.45)					
			Root length (mm) per rooted cutting		
Sorbitol	2.0(1.26)	3.8(1.52)	3.0(1.42)	11.8(3.23)	2.2(1.23)
Sucrose	11.8(3.48)	11.0(3.38)	8.3(2.96)	6.8(2.67)	9.5(3.12)
Glucose	8.3(2.71)	11.8(3.23)	11.3(3.35)	2.5(1.34)	8.5(2.66)
Fructose	0(0.71)	11.5(3.46)	8.5(2.99)	5.8(2.22)	6.5(2.34)
Mean	5.5(2.04)	9.5(2.90)	7.8(2.68)	6.7(2.37)	
LSD (p=0.05) Carbon source, (0.59); Concn., (0.59); Interaction, (1.19)					

[a] Each datum represents the mean of four replications each with five shoot cuttings recorded after two weeks *in vitro*. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

source concentration of 30 and 50 g/L, the concentration influencing these two parameters varied with carbon sources. The highest percent rooting and root number per rooted cutting occurred at concentrations between 10 and 50 g/L for sucrose, 30 and 50 g/L for fructose, 30 g/L for glucose, and no variation among concentrations for sorbitol (Table 13).

With regards to root length per rooted cutting, the longest root length was obtained from cuttings grown on sucrose (9.5 mm) and glucose (8.5 mm) media, intermediate on fructose (6.5 mm), and shortest on sorbitol (2.2 mm) (Table 13). The longest root length per rooted cutting occurred at carbon source concentrations of 10 and 30 g/L for sucrose, 30 and 50 g/L for glucose and fructose, and 70 g/L for sorbitol.

4.4 Discussion and conclusions

Differences in growth and morphological features and in media constituents between M. robusta and Ottawa 3 apple rootstocks during different stages of in vitro propagation were striking. These differences for each in vitro propagation stage of these rootstocks are summarized in Table 14. Although the experimental protocol used in this work does not allow direct interpretation as to why M. robusta reacts differently from Ottawa 3 during various stages of in vitro propagation, nevertheless, the study, for the first time, demonstrates that different apple genotypes have different requirements for carbon sources in in vitro propagation, and also during different stages of the in vitro propagation process. In

TABLE 14. Comparison of differences in shoot proliferation and morphology between Malus robusta No. 5 and Ottawa 3 rootstocks during different stages of in vitro propagation

Stage[a]	Variable	Malus robusta No. 5	Ottawa 3
I	Medium[b]	Nutrient medium + 30 g/L sorbitol + 1.0 mg/L BA + 1.0 mg/L IBA	Nutrient medium + 30 g/L sucrose + 1.0 mg/L BA + 1.0 mg/L NAA
II	Medium[b]	As Stage I	Nutrient medium + 30 g/L sucrose + 0.5 mg/L BA + 0.5 mg/L IBA or NAA + 3.0 mg/L GA
	Culture appearance	Smaller in size; more compact; red pigments present on petioles, stems, and lower leaves in contact with medium	Larger in size; less compact; red pigments absent
	Leaf	Smaller (length = 12-17 mm; width = 5-7mm); less elongated; light green in color	Larger (length = 20-26 mm; width = 7-12 mm); more elongated; green in color
	Stem	Smaller (diameter = 1-1.5 mm)	Larger (diameter = 1.5-2.5 mm)
	Shoot multiplication rate	Higher (six- to nine-fold monthly)	Lower (three- to five-fold monthly)
III	Medium[c]	Rooting medium + 30 g/L sucrose or sorbitol + 0.5 mg/L IBA	Rooting medium + 30 g/L sucrose + 6.25 mg/L IBA
	Time required for maximum rooting	Four weeks	Three weeks

[a] Stages of in vitro propagation, i.e. I, initiation; II, proliferation; III, rooting.

[b] Ingredients are described in Section 3.2.1 but thiourea and asparagine were omitted.

[c] Ingredients are described in Section 3.2.3.

this study, the more rapid growth of meristem tip explants of M. robusta from stoolbed than those from mature tree source seemed to be associated with the greater juvenility of the stoolbed source (Girouard 1974) (see also discussion in Section 5.4). The requirement of sorbitol as a prerequisite for initiation of M. robusta meristem tip cultures and the excellent subsequent in vitro proliferation of these cultures on sorbitol medium clearly demonstrates the importance of sorbitol on growth and development of this genotype in vitro. This confirms work of Chong and Taper (1974a) using callus cultures from the same source.

Meristem tips are small explants and, presumably, the demand for optimal nutrient supply from the medium must be critical. Since explants performed reasonably well on glucose medium, it implies that sorbitol may be metabolized into glucose via direct oxidation by the enzyme SO (Yamaki 1981). However, to optimize the nutrient supply for growth of meristem tips, other pathway(s) of sorbitol degradation may be involved. The most probable pathway seems to be sorbitol$\longrightarrow$S6P. Evidence indicates that S6P is readily converted to G6P in the presence of the enzyme S6PD and NADPH (Negm and Loescher 1981). However, the enzyme responsible for catalysing the reaction, sorbitol$\longrightarrow$S6P, remains to be discovered. Although the pathway of sorbitol$\longrightarrow$fructose may be of importance for sorbitol degradation, particularly in fruits (Yamaki 1980b) and probably in apple callus cultures (Chong and Taper 1974a), results of this study indicate that it is probably not an important pathway for sorbitol degradation during meristem tip growth and shoot proliferation of the M. robusta cultures. Somewhat similar to the occurrence of increased callus growth of M. robusta on fructose medium (Chong and Taper 1972), the marked

tendency for callogenesis and decreased shoot production of the present cultures on fructose medium, suggests that callogenesis occurred, at least in part, at the expense of organogenesis.

In view of the varied response of sugarcane cells to invertase in vitro (Nickell and Maretzki 1970), it is tempting to suggest that the relatively poor growth of M. robusta shoot cultures on sucrose medium may be due to explants which are deficient in invertase. Poor response of sucrose associated with availability of invertase has been suggested by Chong and Taper (1974a) and Maretzki et al. (1974).

In addition to M. robusta, the influence of the various carbon sources on shoot growth of the Ottawa 3 culture is equally striking. The abnormal growth of Ottawa 3 cultures on sorbitol, glucose, and fructose media, and on sucrose medium at concentrations lower or higher than 30 g/L indicates that this abnormality may be associated with sorbitol metabolism. Sorbitol metabolism was closely associated with sugar metabolism (Bieleski 1982). Enzymes responsible for sorbitol synthesis either from glucose or fructose have been isolated from various organs of the apple (Negm and Loescher 1979; 1981; Yamaki 1981). Evidence indicates that sorbitol synthesis could occur via the pathway G6P$\longrightarrow$S6P catalysed by S6PD (Negm and Loescher 1981), and/or directly via reduction of fructose to sorbitol. This reversible reaction is catalysed by the enzyme SDH in the presence of NADH (Negm and Loescher 1979).

Redgwell and Bieleski (1978) have detected S1P and S6P in plum leaves. They postulated that sorbitol synthesis might be via the pathway

F6P⟶S6P. This suggests that abnormal growth of Ottawa 3 cultures grown on glucose and fructose media may be attributed to the conversion of these sugars into sorbitol within plants. In addition, sucrose at high concentrations (> 50 g/L) also may favor sorbitol accumulation. Chong and Taper (1974a) found that up tp 34% sorbitol was detected in apple callus grown on medium supplied with high concentrations of fructose, glucose, and sucrose. Further evidence is provided by Bieleski (1977) that sorbitol was the predominant carbohydrate accumulated in apple tissues after free sugars such as fructose, glucose, and sucrose were supplied to the tissues.

In general, sorbitol enhances stem growth resulting in low leaf/stem ratio (Table 6). The striking stem growth in M. robusta may be attributed to stock cultures which were maintained on sorbitol medium. Sorbitol occurred in relatively high concentrations in the stem compared to the other sugars in vivo (Chong 1971). Since in vitro organogenesis is a high energy-requiring process (Thorpe 1980), perhaps in vitro shoot proliferation is dependent upon high metabolic activities and high demand for energy to sustain these activities in the stem. In comparison with the sugars, sorbitol is more reduced and stores more chemical energy (Wallaart 1980).

In addition to stem growth, the response to different carbon sources also provides an interesting clue to the physiological mechanism of growth compaction in apple. Previous studies in the comparison between spur-type trees and the standard counterpart in vivo indicated that spur-type trees had greater photosynthetic efficiency (Looney 1968),

higher contents of dry weight, nitrogen, calcium, and chlorophyll in leaves (Westwood and Zielinski 1966), and higher mean contents of sorbitol, glucose, and fructose in fruit tissues during the growing season and during storage (Ismail *et al.* 1980). In apple tissue cultures, Lane and Looney (1982) observed more tolerance of shoot cultures to supra-optimal concentrations of cytokinin in more compact-growth type of apple. Spur-type apple trees were characterized by upright growth habit, shorter internodes, fewer but thicker shoots and a higher ratio of reproductive growth (Eaton and Lapins 1970; Lapins 1969; 1974). Cultures with compact growth type grown on sorbitol medium also shared some of these characteristics such as upright growth habit and shorter internodes. This evidence together with results of the present study, indicates that sorbitol metabolism may play an important role in regulation of leaf and stem growth and compactness in shoot cultures of apple. However, the implication between carbohydrate metabolism and growth compaction of apple needs to be further clarified in future studies.

In apple tissue cultures, high percent rooting usually has been obtained in rooting medium supplied with half to two-thirds the concentration of sucrose required for shoot proliferation (Lane 1978; Zimmerman and Broome 1981). This indicates that less carbon source is required for *in vitro* rooting than for shoot proliferation. It is therefore speculated that the amount of sucrose metabolized by shoot cuttings of *M. robusta* may be sufficient for root induction but not shoot proliferation. However, a shift of metabolism resulting in increased synthesis of sucrose-degrading enzymes is also probable. According to

Thorpe (1978), initiation of organized development involves a shift in metabolism in which new enzymes originally absent are synthesized, or enzymes that are present show increased synthesis. In comparison with sucrose, in vitro rooting response of M. robusta on sorbitol medium was slower. It is not clear whether the slower rooting response was due to lower enzyme activity or slower enzyme synthesis. Bieleski (1969) and Grant and Rees (1981) also observed that apple tissues metabolized sorbitol slower than sucrose.

The poor rooting response of Ottawa 3 on sorbitol medium indicates that this rootstock is not capable of metabolizing sorbitol for rooting. The best rooting response on sucrose medium has clearly established the fact that sucrose is the most effective carbon source for in vitro rooting of Ottawa 3 and also M. robusta, due to somewhat earlier root initiation and development in comparison with sorbitol. This result is surprising in view of the fact that roots of apple trees contained relatively high concentration of sorbitol and sorbitol was shown to be actively metabolized particularly at the root apex (Chong 1971; Grant and Rees 1981). Thus, further studies are required to resolve this apparent discrepancy.

CHAPTER V

A COMPARATIVE STUDY ON IN VITRO PROPAGATION OF MACSPUR APPLE FROM SEEDLING AND ADULT PHASES

5.1 Introduction

Ample evidence indicates that clonal propagation of plants is greatly influenced by juvenility. Increasing age or loss of juvenility has been the most important factor affecting rooting of difficult-to-root tree species (Hartmann and Kester 1983; Heuser 1976). Cuttings taken from juvenile plant parts generally rooted more easily than those taken from adult parts of the same plant (Hartmann and Kester 1983).

Callus originating from stem tissues of the juvenile and adult phases of English ivy retained the physiological features of the parent explant for over two years (Stoutemyer and Britt 1965). Evidence also

indicates that, in comparison with cultures originating from juvenile plants, explants taken from adult plants were more difficult to establish, grew poorly in vitro, e.g. blueberry (Lyrene 1978; 1980), and required higher concentrations of auxin for growth of meristem tip explants, e.g. apple (Abbott and Whiteley 1976). Callus derived from embryos and seedlings showed more potential for morphogenesis. Roots, shoots, embryoids, and/or sometimes plantlets have been obtained from juvenile tissues from some woody species (Abbott 1978; Coffin 1974).

Indirect evidence indicates that carbohydrate metabolism may play an important role in juvenility. Clark and Hackett (1980) observed phase reversion of defoliated adult scion grafted onto juvenile stock of English ivy and suggested that phase reversion was attributed to the translocation of ^{14}C-labelled assimilates between partners of juvenile-adult grafts. In apple, more starch and reducing sugars are present in adult wood in comparison with juvenile wood (Doorenbos 1965).

The advantage of using juvenile plant material in plant propagation is well known, but this has not been successfully exploited in plant propagation (Lyrene 1981). This is due primarily to the lack of knowledge in understanding of the physiological bases of juvenility (Borchert 1976). As an alternative approach, plant tissue culture systems seem to offer the greatest promise for gaining more fundamental and theoretical insights into the nature of juvenility. If the nature of juvenility was better understood, then ultimately the effect of developmental phases on cloning may become inconsequential.

The objective of this study was to compare in vitro proliferation and rooting of Macspur (spur-type mutant of McIntosh apple) apple shoot cultures originating from juvenile and adult sources. The comparative requirements of these cultures for different carbon sources, namely, sorbitol, sucrose, glucose, and fructose, during different stages of in vitro propagation were also studied.

5.2 Materials and methods

5.2.1 Seedling phase

5.2.1.1 Initiation

Fruits of Macspur apple collected in the fall of 1979 in the orchard of Macdonald College were stored in a cold room at 4°C for two to three months. Seeds were removed aseptically from fruits surface-sterilized with 95% ethanol. Seed coats were peeled away and naked seeds were placed in 100 x 20 mm petri dishes containing two sheets of sterile-filter paper moistened with sterile-distilled water. Seeded petri dishes were covered with sandwich bags and kept for 7-10 days in the culture room under conditions as described in Section 3.2.1.

Preliminary investigations were conducted to select an appropriate basal medium for shoot proliferation. Shoot tips (ca. 5 mm) taken from aseptically germinated seedlings were transferred to media of Murashige

and Skoog (MS), Shenk and Hildebrandt (SH) (1972), and Uchimiya and Murashige (UM) (1974), which were reported to be successful for culturing apple callus and cell suspension (Lieberman et al. 1979). The concentration of all ingredients in each medium was reduced by 1/8, 1/4, and 1/2 except agar which was added at 7,000 mg/L in all cases. There were eight replications and one explant per treatment in a culture bottle containing 20 ml of medium. In addition, other seedling explants such as cotyledons, hypocotyl and root segments were transferred to petri dishes each containing 40 ml of medium. There were four cotyledons, and six to eight hypocotyl and root segments in each petri dish, with each treatment consisting of four petri dishes.

Growth of shoot tip explants was evaluated after four weeks in terms of number of shoots, shoot length, and shoot fresh weight. Organ and/or callus formation from cotyledons, hypocotyl and root segments also were recorded.

5.2.1.2 Shoot proliferation

Shoots were multiplied and subcultured at monthly intervals on sucrose medium similar to that used for Ottawa 3 rootstock (in Section 3.2.1) but supplemented with 1.0 mg/L BA and 1.0 mg/L IBA. These, and cultures also successfully maintained on sorbitol (30 g/L) medium for three generations (subcultures), were used for all investigations reported herein.

Uniform, single shoot tips (ca. 5 mm) from proliferating cultures maintained on sucrose medium were transferred to similar medium supplemented with either cytokinin (BA, 2ip, kinetin, zeatin), auxin (IBA, NAA, 2,4-D), or combinations of these cytokinins and auxins, each at a concentration of 1.0 mg/L, to select the appropriate growth regulator(s) for the basal culture medium. The experimental method used was similar to that in Section 5.2.1.1. In addition to growth evaluated as in the previous investigation (Section 5.2.1.1), callus formation at the shoot base also was evaluated in terms of callus formation index (CFI) according to the following classification: -, +, ++, and +++ representing no, slight, moderate, and heavy callus formation, respectively.

Investigations on carbon source effect was conducted by transferring shoot tips from sucrose medium to similar medium supplied with sorbitol, sucrose, glucose, and fructose, each at 10, 30, 50, and 70 g/L. In the subsequent investigations, shoot tips taken from cultures maintained on sorbitol and sucrose media were transferred to media supplied with sorbitol and sucrose combinations (30 g/L total) with varying proportions of each, i.e. sorbitol (%) + sucrose (%): 0 + 100, 25 + 75, 50 + 50, 75 + 25, and 100 + 0. In these investigations, there were four replications and five explants per treatment in a 125-ml Erlenmeyer flask containing 30 ml of medium. Shoot proliferation, leaf and stem fresh weight, and leaf/stem ratio were evaluated after four weeks according to procedures described in Section 4.2.1.2. The experiments were repeated and results of the two experiments were pooled.

5.2.1.3 Rooting

In the first investigation, shoot cuttings (2-3 cm) taken from proliferating cultures maintained on sucrose medium were implanted on half-strength (mineral salts) solidifying medium supplemented with 50 mg/L inositol, 30 g/L sucrose, and a series of IBA concentrations (0, 0.1, 0.25, 0.5, 1.0, 3.0, 5.0, and 7.0 mg/L). In the second investigation, cuttings from sucrose medium were transferred to the above rooting medium supplemented with 0.25 mg/L IBA and the four different carbon sources as described for shoot proliferation (Section 5.2.1.2). In the third investigation, cuttings taken from proliferating cultures maintained on sorbitol and sucrose media were implanted in the rooting medium similar to the second investigation but supplied with different sorbitol and sucrose combinations (30 g/L total) as described above. In each investigation, there were four replications and five cuttings per treatment in a 125-ml Erlenmeyer flask containing 40 ml of medium. Rooting response was evaluated after three weeks, and data were transformed as described in Section 3.2.3 before analysed for analysis of variance.

5.2.2 Adult phase

5.2.2.1 Initiation

In May 1981, one-year old shoots of Macspur apple were collected from mature trees (> 10 years old) in the Macdonald College orchard. Shoot bases were immersed in water overnight at room temperature. Techniques used for the initiation of meristem tip cultures were similar to those used for Ottawa 3 rootstock (Section 3.2.1).

5.2.2.2 Shoot proliferation and rooting

Cultures were subsequently multiplied on similar culture media, and subcultured similarly as described for the seedling phase.

All investigations with adult phase material were similar to those in the seedling phase except that preliminary studies with different media (MS, SH, UM) and growth regulators were not conducted. In rooting investigations, rooting response was evaluated after four weeks since adult phase material was slower in rooting.

5.3 Results

5.3.1 Seedling phase

5.3.1.1 Shoot proliferation

Results of the preliminary investigations showed that full-strength (1X) MS medium was most effective for shoot proliferation of seedling phase Macspur. Cultures on this medium produced the highest number of shoots and shoot fresh weight in comparison with SH and UM media, although there was no difference in shoot length among media (Table 15); shoot proliferation generally decreased with increasing nutrient concentrations of all media. Furthermore, 1X MS medium was best in inducing organ formation from cotyledons, hypocotyl and root segments (Table 16). Among these explants, cotyledons (Fig. 9A) and hypocotyl segments (Fig. 9B) gave rise only to adventitious shoots; root segments gave rise only to roots. Organogenesis occurred more frequently from cotyledons (70%) than from other explants.

Table 17 shows the influence of 1X MS medium supplemented with different auxins and cytokinins on proliferation of seedling shoot explants. The highest number of shoots and shoot fresh weight were obtained in treatments containing 1.0 mg/L BA or in combination with either 1.0 mg/L NAA or 1.0 mg/L IBA; differences among growth regulator treatments in terms of shoot length were negligible. All auxins, either alone or in combination with cytokinin, induced callus formation at the

TABLE 15. Shoot proliferation of seedling phase Macspur in response to different nutrient media

Medium	Nutrient[a] concentration	Number of shoots	Shoot length (mm)	Shoot fresh weight (mg)
Murashige and Skoog (MS)	1/8 X	0.5 ± 0[b]	7 ± 1	97 ± 4
	1/4 X	1.3 ± 0.5	11 ± 2	115 ± 8
	1/2 X	2.4 ± 1.1	16 ± 3	388 ± 17
	1 X	3.3 ± 1.0	21 ± 3	465 ± 36
Shenk and Hildebrandt (SH)	1/8 X	0.9 ± 0.4	8 ± 2	103 ± 6
	1/4 X	1.3 ± 0.5	12 ± 2	130 ± 9
	1/2 X	1.9 ± 1.1	16 ± 4	329 ± 15
	1 X	2.3 ± 1.1	21 ± 5	354 ± 27
Uchimiya and Murashige (UM)	1/8 X	0.4 ± 0	5 ± 0	85 ± 2
	1/4 X	0.8 ± 0	10 ± 2	102 ± 5
	1/2 X	1.4 ± 0.2	17 ± 2	338 ± 24
	1 X	2.2 ± 0.4	20 ± 1	376 ± 30

[a] All ingredients of the medium except agar.

[b] Mean ± S.E. of eight explants, one per culture bottle.

TABLE 16. Formation of organs and/or callus from various explant sources of seedling phase Macspur cultures on different nutrient media

Medium	Nutrient[a] concentration	Explant source	Organ formation (%) Shoot	Root	Callus formation (%)
Murashige and Skoog (MS)	1/8 X	Cotyledon	0[b]	0	0
		Hypocotyl	0[c]	0	0
		Root	0[c]	0	0
	1/4 X	Cotyledon	0	0	0
		Hypocotyl	0	0	0
		Root	0	0	0
	1/2 X	Cotyledon	15	0	5
		Hypocotyl	0	0	0
		Root	0	15	10
	1 X	Cotyledon	70	0	30
		Hypocotyl	25	0	90
		Root	0	16	35
Shenk and Hildebrandt (SH)	1/8 X	Cotyledon	0	0	0
		Hypocotyl	0	0	0
		Root	0	0	0
	1/4 X	Cotyledon	0	0	0
		Hypocotyl	0	0	0
		Root	0	0	0
	1/2 X	Cotyledon	0	0	0
		Hypocotyl	0	0	0
		Root	0	0	0
	1 X	Cotyledon	20	0	10
		Hypocotyl	0	0	20
		Root	0	5	8

(table continued)

TABLE 16. (continued)

Medium	Nutrient[a] Concentration	Explant source	Organ formation (%) Shoot	Root	Callus formation (%)
Uchimiya and Murashige (UM)	1/8 X	Cotyledon	0[b]	0	0
		Hypocotyl	0[c]	0	0
		Root	0[c]	0	0
	1/4 X	Cotyledon	0	0	0
		Hypocotyl	0	0	0
		Root	0	0	0
	1/2 X	Cotyledon	5	0	0
		Hypocotyl	0	0	0
		Root	0	5	0
	1 X	Cotyledon	40	0	10
		Hypocotyl	0	0	35
		Root	0	10	20

[a] All ingredients of the medium except agar.

[b] Mean of 16 explants in four petri dish replicates.

[c] Mean of 24-32 explants in four petri dish replicates.

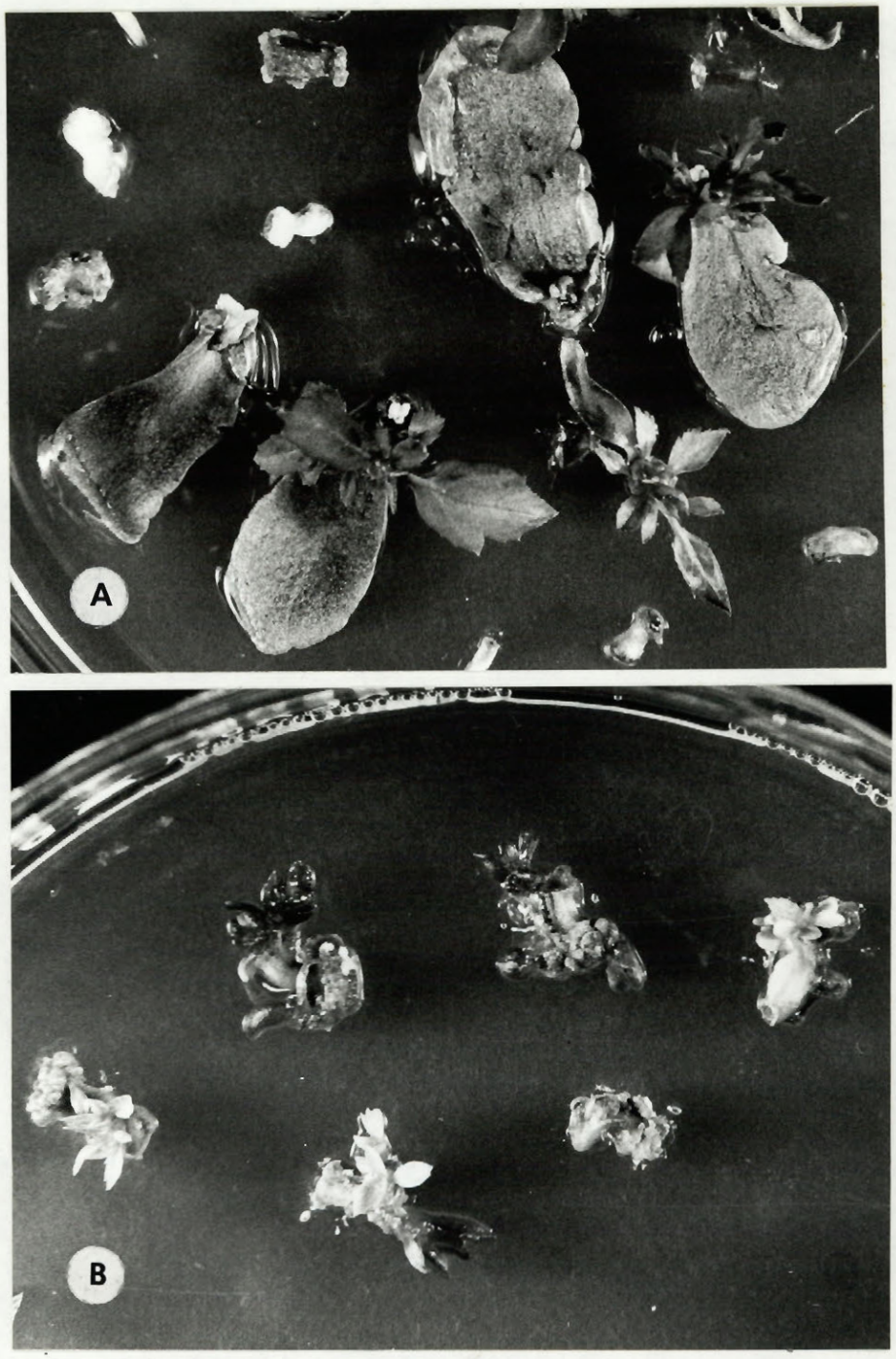


Fig. 9. Development of shoots from cotyledons (A) and hypocotyl segments (B) of seedling phase Macspur apple *in vitro*.

TABLE 17. Shoot proliferation and callus formation of seedling Macspur shoot explants in response todifferent growth regulators in vitro

Growth regulator (1.0 mg/L)	Number of shoots	Shoot length (mm)	Shoot fresh weight (mg)	CFI[a]
BA	5.5 ± 0.8[b]	1.8 ± 0.3	452 ± 35	-
2ip	3.4 ± 0.7	1.7 ± 0.2	419 ± 28	-
Kinetin	1.5 ± 0.8	1.5 ± 0.2	308 ± 27	+
Zeatin	1.1 ± 0.4	1.6 ± 0.1	125 ± 6	+
IBA	1.3 ± 0.5	1.6 ± 0.2	187 ± 10	+
NAA	1.1 ± 0.4	1.4 ± 0.2	142 ± 8	+++
2,4-D	1.3 ± 0.5	1.2 ± 0.1	205 ± 12	+++
BA + IBA	5.2 ± 0.7	1.8 ± 0.2	467 ± 30	+
BA + NAA	5.6 ± 0.9	2.1 ± 0.2	458 ± 28	+
BA + 2,4-D	2.6 ± 0.7	1.8 ± 0.2	385 ± 19	+++
2ip + IBA	2.2 ± 0.5	1.8 ± 0.2	360 ± 21	+
2ip + NAA	1.8 ± 0.7	1.7 ± 0.2	316 ± 23	++
2ip + 2,4-D	1.5 ± 0.8	1.7 ± 0.1	300 ± 18	+++
Kinetin + IBA	1.4 ± 0.5	1.7 ± 0.2	293 ± 21	+
Kinetin + NAA	1.3 ± 0.5	1.6 ± 0.2	263 ± 10	++
Kinetin + 2,4-D	1.8 ± 0.5	1.6 ± 0.1	314 ± 18	+++
Zeatin + IBA	1.4 ± 0.5	1.6 ± 0.2	250 ± 17	+
Zeatin + NAA	1.4 ± 0.5	1.7 ± 0.2	296 ± 12	++
Zeatin + 2,4-D	1.4 ± 0.5	1.7 ± 0.2	240 ± 11	+++

[a] Callus formation index: -, +, ++, and +++ represent no, slight, moderate, and heavy callus formation, respectively.

[b] Mean ± S.E. of eight replicate culture bottles each with one shoot explant.

shoot base. The degree of callus formation on auxin occurred in the following decreasing order: 2,4-D, NAA, and IBA.

Effect of carbon source

Data for shoot proliferation in seedling phase Macspur cultures in terms of number of shoots, shoot length, and shoot fresh weight are presented in Table 18. With regards to carbon source effect, higher number of shoots occurred on sorbitol (5.9) and sucrose (5.7) media compared to glucose (4.4) and fructose (4.3) media. While shoot length on sucrose (16.6 mm) and glucose (15.1 mm) media was significantly higher than on sorbitol (12.4 mm) and fructose (13.4 mm) media, the highest shoot fresh weight occurred on sucrose medium (365 mg). Corresponding shoot fresh weight data for the other carbon sources were in the descending order sorbitol (333 mg), fructose (319 mg), and glucose (301 mg) (Table 18). A significant main effect of concentration, and interaction between carbon source and concentration for all parameters of shoot proliferation also were demonstrated. The highest shoot proliferation occurred at a carbon source concentration of 30 g/L for all carbon sources except sucrose on which shoot proliferation at 30 and 50 g/L was equally effective (Table 18).

Cultures grown on different carbon source media exhibited differential response in leaf and stem fresh weight, and leaf/stem ratio (Table 19). The significant main effect for leaf fresh weight showed that the highest leaf fresh weight occurred on sucrose medium (116 mg); at a concentration of 30 g/L, leaf fresh weight on the other carbon source

TABLE 18. Influence of carbon sources and their concentrations on shoot proliferation of seedling phase Macspur cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Number of shoots			
Sorbitol	3.1[a]	10.1	7.0	2.6	5.9
Sucrose	1.7	8.5	7.8	5.5	5.7
Glucose	2.2	7.0	6.1	2.2	4.4
Fructose	1.6	8.4	5.1	2.2	4.3
Mean	2.1	8.5	6.5	3.6	
LSD (p=0.05)	Carbon source, 0.7; Concn., 0.7; Interaction, 1.4				
		Shoot length (mm)[b]			
Sorbitol	11.6	17.4	12.4	8.3	12.4
Sucrose	9.1	19.4	19.0	18.5	16.6
Glucose	10.0	18.2	16.9	15.1	15.1
Fructose	10.5	14.7	15.5	11.2	13.4
Mean	10.3	17.4	15.9	13.3	
LSD (p=0.05)	Carbon source, 1.4; Concn., 1.4; Interaction, 2.8				
		Shoot fresh weight (mg)			
Sorbitol	240	425	329	336	333
Sucrose	176	469	456	358	365
Glucose	256	396	308	242	301
Fructose	264	433	373	206	319
Mean	234	431	388	286	
LSD (p=0.05)	Carbon source, 29; Concn., 29; Interaction, 58				

[a] Each datum represents the mean of four replications each with five shoots recorded after four weeks *in vitro*.
[b] Length of longest shoot.

TABLE 19. Influence of carbon sources and their concentrations on leaf and stem fresh weight, and leaf/stem ratio of seedling phase Macspur shoot cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Leaf fresh weight (mg)			
Sorbitol	102[a]	94	83	90	87
Sucrose	100	183	101	77	116
Glucose	120	139	105	50	94
Fructose	108	90	90	65	88
Mean	108	126	93	71	
LSD (p=0.05)	Carbon source, 17; Concn., 17; Interaction, 34				
		Stem fresh weight (mg)			
Sorbitol	138	338	267	246	246
Sucrose	76	293	355	282	251
Glucose	134	263	203	192	198
Fructose	156	344	282	141	231
Mean	126	311	277	215	
LSD (p=0.05)	Carbon source, 22; Concn., 22; Interaction, 44				
		Leaf/stem ratio			
Sorbitol	0.74	0.29	0.24	0.36	0.41
Sucrose	1.32	0.61	0.29	0.28	0.62
Glucose	0.91	0.51	0.51	0.26	0.55
Fructose	0.69	0.26	0.33	0.50	0.45
Mean	0.91	0.42	0.34	0.35	
LSD (p=0.05)	Carbon source, 0.09; Concn., 0.09; Interaction, 0.18				

[a]Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

media was not significantly different. Of all carbon sources, leaf fresh weight decreased significantly at concentrations higher than 30 g/L, except for sorbitol on which differences among concentrations were not significant (Table 19). With regards to stem fresh weight, sorbitol (247 mg), sucrose (251 mg), and fructose (231 mg) were significantly higher than glucose (198 mg). A main effect of concentration showed that the highest stem fresh weight occurred at 30 g/L for all carbon sources; however, the highest stem fresh weight for sucrose occurred at 50 g/L. According to data for the main effect of leaf/stem ratio, a significantly lower leaf/stem ratio occurred on sorbitol (0.62) and glucose (0.55) media; leaf/stem ratio at carbon source concentrations between 30 and 70 g/L was significantly lower than that at 10 g/L (Table 19).

Effect of sorbitol and sucrose combinations

Proliferation of shoots derived from cultures maintained on sorbitol and sucrose media in response to sorbitol and sucrose combinations is shown in Table 20. Shoot proliferation in cultures maintained on sorbitol medium was similar to those on sucrose medium. A significant main effect of So + Su was demonstrated for all parameters of shoot proliferation. While cultures in treatment combinations 25 + 75, 50 + 50, 75 + 25, and 100 + 0 showed significantly higher number of shoots (11.8-13.4) than those in 0 + 100 (8.1), the highest shoot length (22.8 mm) and shoot fresh weight (417 mg) occurred in the 25 + 75 combination (Table 20).

Differences between cultures maintained on sorbitol and sucrose

TABLE 20. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on shoot proliferation of seedling phase Macspur shoot cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
			Number of shoots			
Sorbitol	8.1[c]	13.9	12.1	11.9	14.2	11.6
Sucrose	8.0	11.9	11.5	12.6	12.5	11.7
Mean	8.1	12.9	11.8	12.3	13.4	
LSD (p=0.05)	Medium, NS[d]; So + Su, 1.9; Interaction, NS					
			Shoot length (mm)[e]			
Sorbitol	15.5	22.3	20.1	18.2	14.8	18.2
Sucrose	18.9	23.2	20.6	18.8	14.5	19.2
Mean	17.2	22.8	20.4	18.5	14.7	
LSD (p=0.05)	Medium, NS; So + Su, 1.1; Interaction, NS					
			Shoot fresh weight (mg)			
Sorbitol	342	425	386	378	390	384
Sucrose	360	409	379	385	350	379
Mean	351	417	383	382	370	
LSD (p=0.05)	Medium, NS; So + Su, 21; Interaction, NS					

[a]Carbon source medium on which cultures were maintained.

[b]Sorbitol (%) + sucrose (%).

[c]Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[d]Not significantly different.

[e]Length of longest shoot.

media were negligible with regards to leaf and stem fresh weight (Table 21). A significant main effect of So + Su showed that the highest leaf fresh weight occurred in the 25 + 75 combination , while stem fresh weight in 25 + 75, 50 + 50, 75 + 25, and 100 + 0 combinations was significantly higher than that in 0 + 100.

In response to different sorbitol and sucrose combinations, leaf/stem ratio and CI decreased with increasing proportions of sorbitol and decreasing proportions of sucrose in the carbon source fraction (Table 22). Similar to leaf/stem ratio, the main effect of medium for CI was not significantly different; however, it is noteworthy that cultures maintained on sorbitol medium were more compact than those on sucrose medium . Cultures originally maintained on sucrose medium showed similar CI after transferring to media containing high concentrations of sorbitol (50-100%) in the carbon source fraction, but cultures originally maintained on sorbitol medium continued to have significantly lower CI than those on sucrose medium after being transferred to sucrose medium (Table 22).

5.3.1.2 Rooting

Effect of IBA concentrations

Rooting response of shoot cuttings taken from seedling phase Macspur in response to various concentrations of IBA is presented in Table 23. Maximum rooting (100%) and the highest root number per rooted cutting

TABLE 21. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on leaf and stem fresh weight of seedling phase Macspur shoot cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
	Leaf fresh weight (mg)					
Sorbitol	117[c]	148	116	92	99	113
Sucrose	126	139	107	110	62	108
Mean	122	144	112	101	81	
LSD (p=0.05)	Medium, NS[d]; So + Su, 8; Interaction, NS					
	Stem fresh weight (mg)					
Sorbitol	225	277	270	286	286	291
Sucrose	234	270	272	275	291	268
Mean	230	274	271	281	289	
LSD (p=0.05)	Medium, NS; So + Su, 21; Interaction, NS					

[a] Carbon source medium on which cultures were maintained.

[b] Sorbitol (%) + sucrose (%).

[c] Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[d] Not significantly different.

TABLE 22. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on leaf/stem ratio and CI of seedling phase Macspur shoot cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
	Leaf/stem ratio					
Sorbitol	0.55[c]	0.53	0.43	0.32	0.29	0.42
Sucrose	0.54	0.51	0.39	0.39	0.24	0.41
Mean	0.55	0.52	0.41	0.36	0.26	
LSD (p=0.05)	Medium, NS[d]; So + Su, 0.03; Interaction, NS					
	CI[e]					
Sorbitol	0.7	0.7	0.7	0.6	0.4	0.6
Sucrose	1.1	1.1	0.9	0.5	0.3	0.8
Mean	0.9	0.9	0.8	0.6	0.4	
LSD (p=0.05)	Medium, NS; So + Su, 0.3; Interaction, 0.8					

[a]Carbon source medium on which cultures were maintained.

[b]Sorbitol (%) + sucrose (%).

[c]Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[d]Not significantly different.

[e]Compactness index.

TABLE 23. Rooting of seedling phase Macspur apple in response to IBA concentrations after three weeks in vitro

IBA (mg/L)	Rooting (%)	Root number per rooted cutting	Root length (mm) per rooted cutting
0	0(-0.69)[a]	0 (0.71)	0 (0.71)
0.1	60(3.98)	3.6(2.04)	10.1(3.26)
0.25	100(4.61)	9.4(3.15)	8.4(2.99)
0.5	55(4.01)	8.3(2.96)	5.0(2.32)
1.0	0(-0.69)	0 (0.71)	0 (0.71)
3.0	0(-0.69)	0 (0.71)	0 (0.71)
5.0	0(-0.69)	0 (0.71)	0 (0.71)
7.0	0(-0.69)	0 (0.71)	0 (0.71)
LSD (p=0.05)	(0.21)	(0.14)	(0.31)

[a] Each datum represents the mean of four replications each with five shoots cuttings. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

were obtained on medium with 0.25 mg/L IBA; the longest root length per rooted cutting was obtained on medium with 0.1 mg/L IBA. While callus at the base of cuttings was minimal on medium with 0.25 mg/L IBA, callus formation was prominent on medium with IBA between 0.5 and 3.0 mg/L. Callus was not observed on media with 5.0 and 7.0 mg/L IBA.

Effect of carbon source

On media supplied with sorbitol, sucrose, glucose, and fructose, cuttings exhibited marked differential response in terms of percent rooting, root number per rooted cutting, and root length per rooted cutting on these media (Table 24). With regards to the main effects of carbon source and concentration, the highest percent rooting occurred on both sorbitol (81%) and sucrose (78%) media in comparison with that on glucose (45%) and fructose (36%) media, with the optimum mean carbon source concentration at 30 g/L. A significant interaction between carbon source and concentration was also demonstrated. At 30 g/L or higher carbon source concentrations, the variation in percent rooting on sorbitol and sucrose media was not significant, while percent rooting on glucose and fructose media decreased significantly with increasing concentrations (Table 24).

With regards to root number per rooted cutting, main effects showed significantly higher root number per rooted cutting on sorbitol (5.2), sucrose (5.5), and glucose (5.2) media than on fructose medium (3.4), with an optimum mean concentration of 30 g/L. The influence of carbon source concentration for each carbon source was generally similar

TABLE 24. Influence of carbon sources and their concentrations on *in vitro* rooting of seedling phase Macspur apple

Carbon source	Concn. (g/L) 10	30	50	70	Mean
			Rooting (%)		
Sorbitol	50(3.85)[a]	90(4.50)	90(4.50)	95(4.56)	81(4.37)
Sucrose	60(4.08)	100(4.61)	85(4.43)	65(4.18)	78(4.33)
Glucose	70(4.25)	60(4.08)	30(3.29)	20(2.26)	45(3.47)
Fructose	40(3.63)	60(4.08)	40(2.26)	5(0.24)	36(2.55)
Mean	55(3.97)	78(4.32)	61(3.62)	46(2.81)	
LSD (p=0.05) Carbon source, (0.60); Concn., (0.60); Interaction, (1.20)					
			Root number per rooted cutting		
Sorbitol	3.7(2.03)	6.4(2.60)	5.1(2.33)	5.7(2.47)	5.2(2.35)
Sucrose	4.1(2.14)	6.2(2.54)	6.7(2.69)	4.8(2.30)	5.5(2.42)
Glucose	5.4(2.42)	7.9(2.89)	4.3(2.18)	3.2(1.81)	5.2(2.33)
Fructose	4.7(2.28)	5.6(2.41)	2.6(1.66)	0.5(0.93)	3.4(1.82)
Mean	4.5(2.22)	6.5(2.61)	4.7(2.21)	3.6(1.88)	
LSD (p=0.05) Carbon source, (0.27); Concn., (0.27), Interaction, (0.55)					
			Root length (mm) per rooted cutting		
Sorbitol	19.3(3.05)	16.0(4.02)	14.3(3.79)	15.8(3.45)	13.9(3.58)
Sucrose	19.8(4.53)	18.3(4.30)	10.7(2.86)	10.1(2.63)	14.7(3.58)
Glucose	16.0(4.24)	20.1(4.45)	15.9(4.08)	15.2(3.57)	16.8(4.09)
Fructose	17.6(4.28)	19.7(4.49)	16.2(3.70)	5.1(1.67)	14.7(3.54)
Mean	15.7(4.03)	18.5(4.32)	14.3(3.36)	11.6(2.83)	
LSD (p=0.05) Carbon source, (NS[b]); Concn., (0.76); Interaction, (NS)					

[a] Each datum represents the mean of four replications each with five shoot cuttings recorded after three weeks *in vitro*. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

[b] Not significantly different.

to that of percent rooting. There was no difference in root length per rooted cutting among carbon sources; however, a significantly higher root length per rooted cutting occurred at 30 g/L for all carbon sources (Table 24).

Effect of sorbitol and sucrose combinations

Data for *in vitro* rooting in response to different sorbitol and sucrose combinations are shown in Table 25. With regards to the main effect of medium, percent rooting was significantly higher for cuttings taken from cultures maintained on sucrose (92%) than those on sorbitol (33%) medium. Cuttings from different carbon source media also showed different percent rooting in response to sorbitol and sucrose combinations; cuttings from sorbitol medium showed significantly higher percent rooting (40-55%) in the combinations, 25 + 75, 50 + 50, and 75 + 25, while high percent rooting (85-100%) was obtained for cuttings from sucrose medium in all treatment combinations (Table 25).

Similar to percent rooting, significantly higher root number per rooted cutting was obtained for sucrose (6.7) in comparison with sorbitol medium (3.2) (Table 25). Significant main effect of concentration and an interaction between medium and concentration for root number per rooted cutting in different sorbitol and sucrose combinations also were demonstrated. While significantly higher root number per rooted cutting occurred in combinations 50 + 50 and 75 + 25 for cuttings from sorbitol medium (5.5), root number per rooted cutting was high for those from sucrose medium (6.5-7.9) in all treatment combinations except 100 + 0.

TABLE 25. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on *in vitro* rooting of seedling phase Macspur apple cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
			Rooting (%)			
Sorbitol	5(0.95)[c]	45(3.11)	55(3.71)	40(3.81)	20(1.44)	33(2.60)
Sucrose	90(4.50)	85(4.43)	90(4.50)	95(4.56)	100(4.61)	92(4.52)
Mean	48(2.73)	65(3.77)	73(4.11)	68(4.19)	60(3.03)	
LSD (p=0.05)	Medium, (1.33);	So + Su, (0.84);	Interaction, (1.89)			
			Root number per rooted cutting			
Sorbitol	0.8(1.00)	2.6(1.64)	5.5(2.45)	5.5(2.32)	1.8(1.37)	3.2(1.76)
Sucrose	6.5(2.65)	6.5(2.63)	7.9(2.90)	7.4(2.80)	5.4(2.43)	6.7(2.68)
Mean	3.7(1.83)	4.6(2.14)	6.7(2.68)	6.5(2.56)	3.6(1.90)	
LSD (p=0.05)	Medium, (0.49);	So + Su, (0.31);	Interaction, (0.69)			
			Root length (mm) per rooted cutting			
Sorbitol	0.8(1.00)	8.0(2.65)	10.1(3.25)	6.0(2.52)	2.0(1.42)	5.4(2.17)
Sucrose	8.1(2.93)	6.6(2.64)	5.9(2.53)	6.8(2.70)	3.1(1.90)	6.1(2.54)
Mean	4.5(1.97)	7.3(2.65)	8.0(2.89)	6.4(2.61)		
LSD (p=0.05)	Medium, (NS[d]);	So + Su, (0.44);	Interaction, (0.97)			

[a] Carbon source medium on which cultures were maintained.

[b] Sorbitol (%) + sucrose (%).

[c] Each datum represents the mean of four replications each with five shoot cuttings recorded after three weeks *in vitro*. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

[d] Not significantly different.

With regards to root length per rooted cutting, a main effect of concentration, and interaction were significant. Cuttings taken from cultures maintained on sorbitol medium (10.1 mm) exhibited the highest root length in the 50 + 50 combination, while those from sucrose medium (5.9-8.1 mm) showed no significant differences among all treatment combinations except 100 + 0 in which root length was significantly shorter (3.1 mm).

5.3.2 Adult phase

5.3.2.1 Shoot proliferation

Effect of carbon source

Shoot proliferation in response to different carbon sources is presented in Table 26. In the adult phase, number of shoots was approximately half as many as in the seedling phase (Table 18), but the differences between the two growth phases in shoot length and in shoot fresh weight were small. With regards to the main effects of carbon source and concentration in the adult phase, the highest number of shoots occurred on sorbitol medium (3.1), and at carbon source concentrations of 30 and 50 g/L; number of shoots on the other carbon source media was not significantly different. Cultures exhibited similar shoot length on all carbon source media; a significantly higher shoot length occurred at 30 g/L for all carbon sources except for fructose on which the highest was observed at 50 g/L. Although glucose (294 mg) was significantly lower than

TABLE 26 Influence of carbon sources and their concentrations on shoot proliferation of adult phase Macspur cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
		Number of shoots			
Sorbitol	1.6[a]	3.9	3.7	3.2	3.1
Sucrose	1.2	3.1	2.5	2.3	2.3
Glucose	1.2	3.2	2.9	2.2	2.3
Fructose	1.6	3.3	3.2	1.7	2.4
Mean	1.4	3.4	3.1	2.3	
LSD (p=0.05)	Carbon source, 0.4; Concn., 0.4; Interaction, NS[b]				
		Shoot length (mm)[c]			
Sorbitol	12.2	19.0	17.2	13.5	15.5
Sucrose	11.8	20.6	16.0	13.0	15.4
Glucose	12.1	19.4	15.0	13.4	15.0
Fructose	13.1	18.4	20.2	13.5	16.3
Mean	12.3	19.3	17.1	13.4	
LSD (p=0.05)	Carbon source, NS; Concn., 1.2; Interaction, 2.4				
		Shoot fresh weight (mg)			
Sorbitol	349	362	383	260	338
Sucrose	279	415	343	244	320
Glucose	324	402	275	175	294
Fructose	347	361	360	221	322
Mean	325	385	340	225	
LSD (p=0.05)	Carbon source, 25; Concn., 25; Interaction, 50				

[a] Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.
[b] Not significantly different.
[c] Length of longest shoot.

the other carbon sources (320-338 mg) with regards to shoot fresh weight, the highest occurred on sorbitol medium at a concentration of 50 g/L, and at 30 g/L for both sucrose and glucose media (Table 26).

Table 27 shows leaf and stem fresh weight, and leaf/stem ratio in response to different carbon sources. In comparison with the seedling phase (Table 19), there was a tendency for leaf fresh weight and leaf/stem ratio to be higher, and stem fresh weight to be lower in the adult phase (Table 27).

While leaf fresh weight decreased with increasing carbon source concentrations, the main effect of carbon source was not significant (Table 27). Leaves were abnormal in appearance at a concentration of 10 g/L for all carbon sources, as reported in the seedling phase. Stem fresh weight was higher for sorbitol (209 mg), intermediate for sucrose (177 mg) and fructose (179 mg), and least for glucose (156 mg) with regards to carbon source effect. A main effect of concentration showed that the highest stem fresh weight occurred at both 30 and 50 g/L, but this parameter varied with carbon sources with the highest occurring at 50 g/L for sorbitol, 30 g/L for sucrose and glucose, and 30 and 50 g/L for fructose (Table 27). Similar to stem fresh weight, significant main effects and interaction for leaf/stem ratio were also demonstrated. Among carbon sources, sorbitol (0.71) exhibited the lowest leaf/stem ratio, while ratios on the other carbon sources were not significantly different. While leaf/stem ratio was significantly higher at the lowest carbon source concentration (10 g/L), there were no significant differences among concentrations from 30 to 70 g/L for all carbon sources; however, a

TABLE 27. Influence of carbon sources and their concentrations on leaf and stem fresh weight, and leaf/stem ratio of adult phase Macspur shoot cultures

Carbon source	Concn. (g/L) 10	30	50	70	Mean
	Leaf fresh weight (mg)				
Sorbitol	207[a]	120	118	73	130
Sucrose	180	172	142	97	148
Glucose	194	192	99	72	139
Fructose	188	145	133	105	142
Mean	192	157	123	87	
LSD (p=0.05)	Carbon source, NS[b]; Concn., 17; Interaction, 34				
	Stem fresh weight (mg)				
Sorbitol	141	242	265	187	209
Sucrose	99	243	227	139	177
Glucose	130	210	174	112	156
Fructose	159	217	227	116	179
Mean	132	228	223	138	
LSD (p=0.05)	Carbon source, 15; Concn., 15; Interaction, 30				
	Leaf/stem ratio				
Sorbitol	1.49	0.50	0.45	0.39	0.71
Sucrose	1.83	0.71	0.71	0.71	0.99
Glucose	1.51	0.93	0.57	0.64	0.91
Fructose	1.19	0.67	0.59	0.91	0.84
Mean	1.51	0.70	0.58	0.66	
LSD (p=0.05)	Carbon source, 0.12; Concn., 0.12; Interaction, 0.23				

[a] Each datum represents the mean of four replications each with five shoots recorded after four weeks *in vitro*.
[b] Not significantly different.

significantly higher leaf/stem ratio was obtained on glucose medium at 30 g/L and at 70 g/L on fructose medium (Table 27).

Effect of sorbitol and sucrose combinations

Data for shoot proliferation of cultures maintained on sorbitol and sucrose media in response to different sorbitol and sucrose combinations are presented in Table 28. Similar to the previous investigations, the adult phase again showed lower number of shoots than the seedling phase (Table 20); the difference in shoot length and in shoot fresh weight between the two growth phases was less pronounced.

With regards to the main effects of medium and So + Su, a significantly higher number of shoots was obtained for cultures grown on sorbitol (7.4) in comparison with those on sucrose medium (4.3). Number of shoots in the combination of 0 + 100 was significantly lower, while differences among other combinations were negligible (Table 28). There was no difference in shoot length and in shoot fresh weight between cultures from sorbitol and sucrose media. A significant So + Su effect showed that shoot length tended to decrease with increasing proportion of sorbitol and with decreasing proportion of sucrose in the carbon source fraction; the highest shoot fresh weight for cultures maintained on sorbitol medium occurred in the 75 + 25 combination, while those on sucrose medium occurred in the 50 + 50 combination (Table 28).

Table 29 shows leaf and stem fresh weight of cultures grown on media supplied with different sorbitol and sucrose combinations. A higher

TABLE 28. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on shoot proliferation of adult phase Macspur cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
			Number of shoots			
Sorbitol	4.5[c]	7.9	8.3	8.0	8.1	7.4
Sucrose	1.9	3.8	5.9	4.6	5.1	4.3
Mean	3.2	5.9	7.1	6.3	6.6	
LSD (p=0.05)	Medium, 2.2; So + Su, 1.4; Interaction, NS[d]					
			Shoot length (mm)[e]			
Sorbitol	17.8	18.5	14.5	15.1	15.6	16.3
Sucrose	16.0	16.0	17.8	16.6	14.3	16.1
Mean	16.9	17.3	16.2	15.9	15.0	
LSD (p=0.05)	Medium, NS; So + Su, 0.9; Interaction, NS					
			Shoot fresh weight (mg)			
Sorbitol	372	365	358	406	349	370
Sucrose	308	368	410	388	367	367
Mean	335	367	384	397	358	
LSD (p=0.05)	Medium, NS; So + Su, 14; Interaction, 32					

[a]Carbon source medium on which cultures were maintained.

[b]Sorbitol (%) + sucrose (%).

[c]Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[d]Not significantly different.

[e]Length of longest shoot.

TABLE 29. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on leaf and stem fresh weight of adult phase Macspur shoot cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
			Leaf fresh weight (mg)			
Sorbitol	155[c]	130	127	134	111	131
Sucrose	145	138	131	126	120	132
Mean	150	134	129	130	116	
LSD (p=0.05)	Medium, NS[d]; So + Su, 12; Interaction, NS					
			Stem fresh weight (mg)			
Sorbitol	217	235	231	273	238	239
Sucrose	163	230	279	264	247	237
Mean	190	233	255	269	243	
LSD (p=0.05)	Medium, NS; So + Su, 14; Interaction, 32					

[a] Carbon source medium on which cultures were maintained.

[b] Sorbitol (%) + sucrose (%).

[c] Each datum represents the mean of four replications each with five shoots recorded after four weeks *in vitro*.

[d] Not significantly different.

leaf fresh weight and lower stem fresh weight in the adult than in the seedling phase again were demonstrated (Table 21). While a significant main effect of So + Su showed that leaf fresh weight tended to decrease with increasing proportion of sorbitol and with decreasing proportion of sucrose in the carbon source fraction, there was no difference in leaf and in stem fresh weight between cultures maintained on sorbitol and sucrose media; the highest stem fresh weight occurred in the 75 + 25 combination of for those cultures from sorbitol medium, but in 50 + 50 for those from sucrose medium (Table 29).

Cultures grown on media supplied with different sorbitol and sucrose combinations exhibited differential response in leaf/stem ratio and CI (Table 30). In comparison with the seedling phase (Table 21), both leaf/stem ratio and CI tended to be higher in the adult phase. A significant main effect of So + Su showed that both leaf/stem ratio and CI decreased with increasing sorbitol and with decreasing sucrose proportions in the carbon source combinations; however, those cultures maintained on sucrose medium exhibited similar CI in all treatment combinations except 100 + 0 (Table 30).

Similar to the seedling phase, the adult phase cultures maintained on sorbitol medium were more compact than those maintained on sucrose medium. While cultures originally maintained on sorbitol medium showed similar CI after transferring to sucrose medium (0 + 100) but not other treatment combinations, cultures maintained on sucrose medium exhibited a significantly higher CI than those on sorbitol medium after being transferred to sorbitol medium (Table 30).

TABLE 30. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on leaf/stem ratio and CI of adult phase Macspur shoot cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
	Leaf/stem ratio					
Sorbitol	0.72[c]	0.56	0.55	0.50	0.48	0.56
Sucrose	0.66	0.60	0.54	0.48	0.49	0.55
Mean	0.69	0.58	0.55	0.49	0.49	
LSD (p=0.05)	Medium, NS[d]; So + Su, 0.04; Interaction, NS					
	CI[e]					
Sorbitol	2.5	1.4	0.8	0.6	0.6	1.2
Sucrose	2.3	1.9	2.1	2.1	1.2	1.9
Mean	2.4	1.7	1.5	1.4	0.9	
LSD (p=0.05)	Medium, NS; So + Su, 0.5; Interaction, 1.0					

[a]Carbon source medium on which cultures were maintained.

[b]Sorbitol (%) + sucrose (%).

[c]Each datum represents the mean of four replications each with five shoots recorded after four weeks in vitro.

[d]Not significantly different.

[e]Compactness index.

5.3.2.2 Rooting

Effect of IBA concentrations

Rooting of adult phase Macspur shoot cuttings on media supplemented with different concentrations of IBA is presented in Table 31. Unlike the seedling phase in which the lower IBA concentration (0.25 mg/L) and shorter time (three weeks) were required for 100% rooting (Table 23), percent rooting in the adult phase increased progressively with increasing IBA concentrations between 0 and 3.0 mg/L, with 100% rooting obtained at 3.0 mg/L after four weeks (Table 31). There was no difference in root number per rooted cutting in response to IBA concentrations higher than 0.5 mg/L, but root length per rooted cutting tended to be higher at lower (0.25-3.0 mg/L) than higher (5.0-7.0 mg/L) IBA concentrations.

Effect of carbon source

With regards to carbon source effect, rooting response of the adult phase also was different from that of the seedling phase in which both sorbitol and sucrose were the most effective carbon sources for rooting (Table 24). In the adult phase, sucrose (86%) was the most effective carbon source for rooting; fructose (63%) and glucose (53%) were intermediate, and sorbitol (20%) was least effective (Table 32). The highest percent rooting occurred at 30 g/L for all carbon sources. The significant main effects of carbon source and concentration showed that

TABLE 31. Rooting of adult phase Macspur apple in response to IBA concentrations after four weeks in vitro

IBA (mg/L)	Rooting (%)	Root number per rooted cutting	Root length (mm) per rooted cutting
0	0(-0.69)[a]	0 (0.71)	0 (0.71)
0.1	0(-0.69)	0 (0.71)	0 (0.71)
0.25	15(2.09)	2.3(1.44)	6.1(2.55)
0.5	40(3.63)	3.6(2.01)	6.3(2.61)
1.0	75(4.32)	5.5(2.43)	6.2(2.59)
3.0	100(4.61)	6.8(2.69)	5.6(2.47)
5.0	85(4.44)	5.4(2.43)	5.6(2.47)
7.0	55(3.91)	5.6(2.47)	1.0(1.21)
LSD (p=0.05)	(1.05)	(0.86)	(0.50)

[a] Each datum represents the mean of four replications each with five shoots cuttings. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

TABLE 32. Influence of carbon sources and their concentrations on *in vitro* rooting of adult phase Macspur apple

Carbon source	Concn. (g/L) 10	30	50	70	Mean
			Rooting (%)		
Sorbitol	15(0.51)[a]	40(3.70)	15(2.09)	10(1.17)	20(1.87)
Sucrose	75(4.31)	100(4.61)	90(4.50)	80(4.38)	86(4.45)
Glucose	55(4.01)	80(4.38)	55(3.86)	20(2.26)	53(3.63)
Fructose	70(4.25)	85(4.45)	75(4.31)	20(2.26)	63(3.82)
Mean	54(3.27)	76(4.28)	59(3.69)	33(2.52)	
LSD (p=0.05)	Carbon source, (0.53); Concn., (0.53); Interaction, (NS[b])				
			Root number per rooted cutting		
Sorbitol	0.8(1.00)	1.5(1.28)	1.5(1.35)	0.5(0.97)	1.1(1.15)
Sucrose	3.7(2.04)	4.6(2.25)	5.1(2.36)	5.2(2.37)	4.7(2.26)
Glucose	3.8(2.07)	4.5(2.23)	3.0(1.86)	1.0(1.18)	3.1(1.84)
Fructose	6.1(2.56)	5.2(2.36)	3.7(2.03)	3.4(1.84)	4.6(2.20)
Mean	3.6(1.92)	4.0(2.03)	3.3(1.90)	2.5(1.59)	
LSD (p=0.05)	Carbon source, (0.29); Concn., (0.29), Interaction, (NS)				
			Root length (mm) per rooted cutting		
Sorbitol	2.8(1.38)	6.3(2.16)	6.8(2.45)	10.3(2.57)	6.6(2.14)
Sucrose	16.0(3.99)	19.0(4.37)	17.8(4.26)	12.3(3.56)	16.3(4.05)
Glucose	16.0(4.05)	20.0(4.47)	12.0(3.53)	8.8(2.79)	14.2(3.71)
Fructose	17.8(4.27)	21.0(4.63)	19.8(4.48)	9.3(2.86)	17.0(4.06)
Mean	13.2(3.42)	16.6(3.91)	14.1(3.68)	10.2(2.95)	
LSD (p=0.05)	Carbon source, (0.76); Concn., (NS); Interaction, (NS)				

[a]Each datum represents the mean of four replications each with five shoot cuttings recorded after four weeks *in vitro*. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysisof variance.

[b]Not significantly different.

at concentrations between 10 and 50 g/L, the highest root number per rooted cutting occurred on both sucrose (4.7) and fructose (4.6) media in comparison with glucose (3.1) and sorbitol (1.1) media. With regards to root length per rooted cutting, sucrose (16.3 mm), glucose (14.2 mm), and fructose (17.0 mm) were the highest; root length per rooted cutting at all carbon source concentrations was not significantly different (Table 32).

Effect of sorbitol and sucrose combinations

In rooting media supplied with different sorbitol and sucrose combinations, equally high percent rooting was obtained in cuttings taken from cultures maintained on sorbitol (94%) and sucrose (87%)media (Table 33). This contrasts with the seedling phase in which high percent rooting occurred only in those from sucrose medium (Table 25). A significant So + Su effect and interaction showed that high percent rooting of cuttings from both media occurred in all treatment combinations except 100 + 0 in which cuttings from sucrose medium were poorly rooted (50%). With regards to the siginificant main effects for root number per rooted cutting, cuttings from sucrose medium (9.2) were higher than those from sorbitol medium (7.1); the highest root number per rooted cutting occurred in treatment combinations similar to percent rooting. While the longest root length per rooted cutting occurred in the 25 + 75 combination, there were no significant diferences between cuttings taken from cultures maintained on sorbitol and sucrose media (Table 33).

TABLE 33. Influence of sorbitol and sucrose combinations (30 g/L total concentration) on in vitro rooting of adult phase Macspur apple cultures maintained on different carbon source media

Medium[a]	So + Su[b]					Mean
	0 + 100	25 + 75	50 + 50	75 + 25	100 + 0	
			Rooting (%)			
Sorbitol	80(4.39)[c]	100(4.61)	100(4.61)	100(4.61)	90(4.50)	94(4.54)
Sucrose	95(4.56)	100(4.61)	95(4.56)	95(4.56)	50(3.91)	87(4.44)
Mean	88(4.48)	100(4.61)	98(4.59)	98(4.59)	70(4.21)	
LSD (p=0.05)	Medium, (NS[d]); So + Su, (0.16); Interaction, (0.38)					
			Root number per rooted cutting			
Sorbitol	5.2(2.38)	7.5(2.82)	7.5(2.82)	10.2(3.26)	5.1(2.35)	7.1(2.73)
Sucrose	7.8(2.86)	9.5(3.16)	9.9(3.21)	9.7(3.19)	7.3(2.78)	9.2(3.11)
Mean	6.5(2.62)	8.5(2.99)	8.7(3.02)	10.0(3.23)	6.2(2.57)	
LSD (p=0.05)	Medium, (0.34); So + Su, (0.22); Interaction, (NS)					
			Root length (mm) per rooted cutting			
Sorbitol	20.3(4.55)	21.3(4.66)	21.3(4.89)	18.3(4.32)	18.5(4.35)	19.9(4.55)
Sucrose	19.3(4.45)	24.8(5.25)	18.5(4.36)	15.8(4.00)	16.0(4.05)	18.9(4.42)
Mean	19.8(4.50)	23.1(4.96)	19.9(4.63)	17.1(4.16)	17.3(4.20)	
LSD (p=0.05)	Medium, (NS); So + Su, (0.22); Interaction, (NS)					

[a]Carbon source medium on which cultures were maintained.

[b]Sorbitol (%) + sucrose (%).

[c]Each datum represents the mean of four replications each with five shoot cuttings recorded after four weeks in vitro. All values not in parenthesis are means of untransformed data. All values in parenthesis are means of transformed data used for analysis of variance.

[d]Not significantly different

5.4 Discussion and conclusions

In this study, the cultures of Macspur apple originating from seedlings and adult trees exhibited striking differences in shoot proliferation and morphology. These comparative differences during each stage of *in vitro* propagation are summarized in Table 34.

In the literature, young seedlings have been used as the juvenile source for comparative studies on the growth phases of apple *in vitro* (Abbott and Whiteley 1976; Welander and Huntrieser 1981). In the present study, the Macspur cultures originating from seedlings did exhibit juvenility by its greater vegetative regeneration ability. The disadvantage of using seedlings is that they are not true-to-type in comparison with mature fruit trees derived from grafting. Although juvenile conditions also can be derived from adventitious shoots of stoolbeds, or those caused to revert to juvenile phase by methods such as gibberellin treatment, grafting to juvenile wood (Hartmann and Kester 1983), or use of adventitious shoots arising from stems or roots (Stoutemyer 1937), the degree of juvenility can be expected to be somewhat lower or variable in comparison with that of seedlings (Stoutemyer and Britt 1969). Thus, in the present study, the ensured juvenility of seedling material is a major advantage.

In plant tissue cultures, spontaneous rejuvenation observed on

TABLE 34. Comparison of differences in shoot proliferation and morphology between the seedling and adult phases of Macspur apple during different stages of *in vitro* propagation

Stage[a]	Variable	Seedling phase	Adult phase
I	Medium[b]	Nutrient medium + 30 g/L sucrose + 1.0 mg/L BA + 1.0 mg/L IBA	As seedling phase
II	Medium[b]	As Stage I	As Stage I
	Culture appearance	More compact	Less compact
	Leaf	Smaller (length = 10-15 mm; width = 5-8 mm); strongly serrated; less elongated; dark green in color	Larger (length = 15-25 mm; width = 7-12 mm); less serrated; more elongated; light green in color
	Stem	Smaller (diameter = 1-1.5 mm); shorter internodes	Larger (diameter = 1.5-2.5 mm); longer internodes
	Shoot multiplication rate	Higher (five- to eight-fold monthly)	Lower (three- to five-fold monthly)
III	Medium[c]	Rooting medium + 30 g/L sucrose + 0.25 mg/L IBA	Rooting medium + 30 g/L sucrose + 3.0 mg/L IBA
	Time required for maximum rooting	Three weeks	Four weeks
	Root appearance	Smaller; less secondary roots; greater pigmentation after prolonged culture	Larger; more secondary roots; lesser pigmentation after prolonged culture

[a] Stages of *in vitro* propagation, i.e. I, initiation; II, proliferation; III, rooting.

[b] Ingredients are described in Section 3.2.1 but thiourea and asparagine were omitted.

[c] Ingredients are described in Section 3.2.3.

adult explants after several subcultures, e.g. grape (Mullins et al. 1979) and blueberry (Lyrene 1981), may become an alternative means of obtaining juvenile shoots. However, cultures initiated from both juvenile and adult phase materials in the present study appeared to be stable on the basis of their morphological features and shoot proliferation after two years of continuous subculturing. Results also showed that Macspur apple from both juvenile and adult phases can be easily rooted in vitro although the optimum requirements for auxin was different for each phase. Rooted shoots of both growth phases acclimatized well after transfer from agar medium to soil under humid greenhouse conditions.

Enhanced ability of vegetative regeneration has been one of the important characteristics of juvenility in woody plants (Hartmann and Kester 1983). The differences in vegetatively regenerative ability between juvenile and adult plants not only commonly occurred in vivo but also in vitro. For instance, shoot tip cultures of blueberry derived from juvenile shoots grew more rapidly and vigorously than those from adult shoots (Lyrene 1978; 1980). In Macspur apple, the more rapid shoot proliferation in seedling cultures can be attributed to cultures which are more juvenile in nature compared to those initiated from mature trees. According to Abbott and Whiteley (1976), growth differences between juvenile and adult cultures of Cox's Orange Pippin apple in vitro may be associated with differences in relative level of endogenous growth regulators; they observed more growth and development in juvenile cultures than those in adult cultures on medium in the absence of auxin. Other

studies also indicated the probability of relatively high endogenous auxin content in juvenile tissues. For instance, Lane (1978) found that the presence of auxin in the medium inhibited shoot proliferation of McIntosh apple. In the present study, callus formation has usually been observed at the shoot base of the juvenile cultures grown on shoot proliferation medium supplemented with 1.0 mg/L BA and 1.0 mg/L IBA, while callus formation was not observed in adult cultures on similar medium. Since callus formation can be readily induced by using supra-optimal auxin concentrations in the medium (Hammerschlag 1982a), this observation appears to support the hypothesis that juvenile tissues contain sufficient endogenous auxin for growth in vitro.

Carbohydrate may play an important role in vitro organogenesis, as discussed previously in Section 4.4. Results of this study indicate that juvenile as well as adult cultures can utilize either sorbitol, sucrose, glucose, or fructose as a sole carbon source for shoot proliferation in vitro, although sorbitol was more effective for this genotype. The greater shoot proliferation in juvenile cultures can at least partially be explained by the hypothesis that in these cultures sorbitol is synthesized via hexose phosphates derived from sucrose degradation or other sugars such as glucose and fructose occurring in leaves and stems. Evidence indicates that in apple and other plants of the Rosaceae, sorbitol was the major metabolite of photosynthesis (Chong 1971), and thus its synthesis occurred mainly in leaves in vivo (Grant and Rees 1981). Since callus, leaves or other chlorophyll-containing tissues undergo photosynthesis at a reduced level in plant tissue cultures (Chong and Taper 1974b; Hughes 1981b), sorbitol synthesis can occur in

photosynthesizing leaves in apple tissue cultures at a reduced rate. According to Hoflacher and Bauer (1982), the high photosynthetic rate in English ivy was associated with high chloroplast per unit leaf area and thicker leaves. This indicates that leaves of juvenile phase Macspur cultures may undergo a higher rate of photosynthesis in vitro resulting in higher sorbitol content; preliminary investigations showed that leaf chlorophyll content in juvenile cultures was higher than that in the adult cultures, particularly when the medium was supplied with either sorbitol or sucrose. In addition to leaves, stem tissues of apple seedlings are also capable of synthesizing or degrading sorbitol in vitro (Grant and Rees 1981). However, mature tree stem tissues seem to lack the ability for sorbitol synthesis and degradation (Bieleski 1969). Therefore, evidence from these studies suggest that sorbitol synthesis, if any in adult cultures, probably occurred at a much lower rate compared to juvenile cultures in vitro.

While it is not surprising that both juvenile and adult phase cultures of Macspue apple can be maintained on sorbitol medium, the marked increase in number of shoots and compact growth occurring in both juvenile and adult cultures grown on sorbitol medium in comparison with those on sucrose medium confirms results of Section 4.3.1.1. These growth habits not only persisted on sorbitol medium up to four generations (no growth determination was attempted after four generations), but, it is

noteworthy that number of shoots of adult cultures grown on sorbitol medium for four generations was further increased 1.6-fold over those grown on the same medium for one generation. This resulted in a total increase of four-fold compared to corresponding sucrose-grown cultures (Table 28). Although enhanced shoot proliferation rate of apple has been achieved by growing shoot tip cultures on medium supplemented with supra-optimal concentrations of cytokinin (3-5 mg/L BA), the resulting shoots were stunted (Lundergan and Janick 1980). In the present study, adult cultures maintained on sorbitol medium appeared very compact and contained abundant small shoots but, more importantly, these shoots were normal in appearance. When shoot tips were re-cultured on sucrose medium, length of internodes and mean shoot length of both juvenile and adult cultures were observed to increase even though length of the longest shoot and shoot fresh weight were not affected. Furthermore, number of shoots tended to decrease and cultures appeared less compact. This type of shoot proliferation was similar to that maintained on sucrose medium. This finding is of significance in in vitro propagation of apple since number of shoots in cultures maintained on sorbitol medium can be increased by two- to four-fold over standard nutrient medium with sucrose.

The above results suggest that a partial reversion to juvenility in apple tissue cultures may be related to the presence of sorbitol. Evidence from the literature indicates that the juvenile and adult tissues of apple exhibited differential ability to convert sucrose to sorbitol. According to Yamaki (1980a), apple seedlings were able to synthesize

sorbitol by using phosphate derivatives of glucose and fructose which were produced from degradation of sucrose and other sugars. On the other hand, radioactive tracer study in apple seedlings showed that sorbitol and sucrose were equally labelled quantitatively (Grant and Rees 1981). This indicates that both sorbitol and sucrose probably are the predominate carbohydrates accumulated in juvenile tissues.

In contrast to juvenile tissues, evidence indicates that sucrose was the predominant carbohydrate constituent in apple callus tissues initiated from adult phase meterial (Chong and Taper 1974a), and was the major sugar accumulated in the phloem tissue after sucrose was supplied exogenously to the tissue (Bieleski 1969). This evidence, together with results of the present study, imply that the lower vegetative regeneration ability in adult cultures maintained on sucrose medium may be attributed to the tissue lacking ability to convert sucrose to sorbitol, and that sucrose probably is the predominate carbohydrate acumulated in these tissues. A remarkable increase in vegetatively regenerative ability resembles the phenomenon of culture rejuvenation occurring after cultures were grown on sorbitol medium for four generations. This "rejuvenation" effect is probably associated with increased synthesis of sorbitol-degrading enzymes such as SDH (Loescher et al. 1982; Negm and Loescher 1981; Yamaki 1980b) and SO (Yamaki 1980b;1982a) in cultures maintained on sorbitol medium. However, further studies are needed to clarify this point.

Many woody plants are difficult to root especially when they grow

older. It has long been advised that high concentrations of growth regulators might promote rooting in these plants (Hartmann and Kester 1983). A favorable use of high IBA conentrations between 10,000 and 40,000 ppm has successfully stimulated rooting of stem cuttings of certain difficult-to-root species (Burd and Dirr 1977; Brown and Dirr 1976; Chong 1981). Although relatively high auxin concentration treatment can markedly enhance rooting, the process of adventitious root formation occurred more readily in juvenile than adult cuttings. For instance, maximum rooting of creeping fig occurred after 17 days for juvenile cuttings and 28 days for adult cuttings (Davies et al. 1982). In blueberry, maximum percent rooting in juvenile shoot cuttings was higher and occurred more rapidly compared to that in adult shoot cuttings (Lyrene 1981). A similar phenomenon also was observed in in vitro rooting of Macspur apple in this study. The juvenile cultures possessed significantly higher shoot proliferative capacity and contained more abundant shoot tips; this may have resulted in higher endogenous auxin content in the juvenile cuttings since the usual site of auxin formation is located in meristem and enlarging tissues (Leopold and Krisdemann 1975), and may explain why seedling cuttings required less endogenous auxin to obtain maximum rooting. In addition, juvenile and adult plants usually resemble easy-to-root and difficult-to-root species, respectively, in terms of rooting ability. It is therefore suggested that difference in auxin metabolism also may be the contributory factor for rooting differences between the growth phases. James (1983b) suggested this hypothesis to account for differences in rooting of the difficult-to-root apple

rootstock M.9 and easy-to-root M.26 *in vitro*. However, other possibilities cannot be excluded. For instance, juvenile plants may contain root-promoting substances, e.g. *M. robusta* (Quamme and Nelson 1965), an increase in root inhibitor production as the plant grows older e.g. *Eucalyptus* spp. (Paton *et al.* 1970), lesser amount of phloem fiber, e.g. Ottawa 3 apple rootstock (Nelson 1978), or higher levels of polyphenolic oxidase enzyme (Bassuk *et al.* 1981), and phenolic compounds such as phloridzin in apple, which were postulated as auxin co-factor or synergists in root initiation (Hartmann and Kester 1983).

The amount of carbohydrates present in the cuttings greatly influenced rooting of apple and other woody plants. For instance, the amount of starch content in stem cuttings of grape was positively correlated to percent rooting (Winkler 1927). Under *in vivo* conditions, carbohydrate has been considered to affect only root development since carbohydrate could not effectively be added to cuttings (Tukey 1979); however, as yet, there is no appropriate means for evaluating the influence of carbohydrate on root initiation *in vivo* (Tukey 1979). Using plant tissue culture techniques, this study clearly demonstrates that the kind and concentrations of carbohydrates not only affect root development but also root initiation in shoot cuttings of both juvenile and adult phases.

In the juvenile cultures maintained on sucrose medium, the ease of rooting in all sorbitol and sucrose combinations further indicates that

the juvenile tissues are capable of utilizing effectively both sorbitol and sucrose. However, the cause of poor rooting response in cuttings of the juvenile phase taken from cultures maintained on sorbitol medium is not clear. In addition to juvenile cultures, sucrose is shown to be essential for maximum rooting in the adult cuttings from cultures maintained on sucrose medium, but for those cuttings from sorbitol medium, maximum rooting was obtained as easily as the juvenile cuttings from sucrose medium. Further studies such as with radioactive tracers are needed to elucidate this discrepancy in utilization of sorbitol by juvenile and adult tissues for in vitro rooting.

CHAPTER VI

CELL SUSPENSION CULTURES OF APPLE

6.1 Introduction

Somatic embryogenesis via cell suspension culture possesses the greatest potential for rapid clonal propagation of plants (Murashige 1978; Winton 1978). Steward et al. (1958) were the first to produce plants from single cells in suspension culture. This achievement demonstrated that each living cell, somatic or otherwise, possesses the inherent capacity to form a complete organism when isolated from the confounding influences of adjacent tissues and provided with the appropriate biochemical stimuli.

To date somatic embryogenesis in cell suspension culture is limited to herbaceous species (Abbott 1978; Mott 1981). Although suspension cultures have been reported for apple including young (Thorpe

1978) and post-climacteric (Lieberman et al. 1979; Pech et al. 1974) fruit tissues, and from tissues of stem tissues derived from this laboratory (Coffin 1974), and a number of other woody species (Abbott 1978), somatic embryogenesis has not been reported. However, Durzan and Lopushanski (1975) grew cells of American elm for five months as cell suspension, which when plated out on agar medium regenerated shoots or roots.

The requirements for single cell growth appear to be more stringent than for organ or callus cultures, and the present situation reflects inadequate knowledge in this area. However, the literature suggests that induction of somatic embryogenesis may be influenced by plant growth substances and nutrients in the culture medium (Sharp et al. 1980; Thorpe 1982; Vasil and Vasil 1980). In certain cases, some ingredients in the medium such as activated charcoal (Litz and Conover 1980) or coconut milk (Litz and Conover 1982) were prerequisites for inducing a high frequency of embryogenic cells.

The objectives of this study were to establish a cell suspension culture of apple, and to attempt to induce somatic embryogenesis for mass propagation of Malus.

6.2 Materials and methods

6.2.1 Callus culture

Shoot tips (ca. 3 mm) of Macspur apple seedlings were obtained from established in vitro shoot proliferating cultures (in Section 2.2.1) and transferred to 60 x 15 mm petri dishes each containing 5 ml of modified MS medium, described as below, supplemented with different combinations of BA and NAA each at 0, 0.1, 0.5, 1.0, and 2.0 mg/L. Petri dishes were placed in polyethylene bags and kept in darkness at room temperature. Callus formation was evaluated in terms of callus formation index (CFI) as described in Section 5.2.1 after six weeks.

The culture medium consisted of MS macro- and micro-elements with iron added as 30 mg/L $FeNa_2EDTA$ and the following constituents (mg/L): thiourea, 25; asparagine, 180; inisitol, 100; calciumm pantothenate, 1.0; nicotinic acid, 1.0; thiamin.HCl, 1.0; pyridoxin.HCl, 1.0; biotin, 0.01; sucrose, 30,000; Difco-bacto agar, 7,000. The pH of the medium was adjusted to 5.7 before autoclaving.

Callus cultures of adult phase Macspur apple were initiated as those of seedlings on similar medium but supplemented wth 0.5 mg/L BA and 2.0 mg/L NAA.

Calli from seedling and adult phases were separated from the parent tissues after six weeks, and thereafter subcultured under

conditions described above at monthly intervals on medium similar to that used for callus growth of adult phase material.

Attempts were made to induce callus formation from nucellus tissues of young Macspur fruits (1-2 cm) according to the method described by Eichholtz et al. (1979b) but the tisuues failed to grow on the recommended medium also on the medium used for callus growth in this study.

6.2.2 Cell suspension culture

Cell suspension cultures from seedling and adult phase materials were initiated as followed: a mass of friable callus tissue, approximately 500 mg, was aseptically transferred to 125-ml Erlenmeyer flask containing 25 ml of liquid medium. Cultures were continuously shaken at 120 r.p.m. (Eberbach Coop., Ann Arbor, Michigan) at 26 ± 2°C and 16-hour photoperiod of 51.5 $\mu E\ s^{-1}\ m^{-2}$.

6.2.2.1 Culture medium

Suspension cultures used for all investigations in this Section were initiated from callus cultures of the seedling phase. The first investigation was conducted by using liquid medium of similar composition

described for callus growth but supplemented with 1.0 mg/L BA and 1.0 mg/L NAA. This was referred to as the M1 medium. Two flasks of cultures were harvested at weekly intervals to determine growth of cell suspension in terms of cell number and fresh weight against time course. In the second investigation, cell suspension was grown on the following media: M1; Shenk and Hildebrandt (SH); Uchimiya and Murashige (UM). There were 10 replicate flasks for each medium. The third investigation was conducted using similar medium with the macro-elements at full-strength (M1) or reduced by 1/2 (M2), 1/3 (M3), 1/5 (M5), 1/7 (M7), or 1/9 (M9). There were six replicate flasks for each treatment. Growth of cell suspension in the second and third investigations was measured in terms of cell number, packed cell volume (PCV), and fresh weight after four weeks.

Cell number was determined by counting with the aid of a hemacytometer after one volume of cell suspension was mixed with two volumes of 4% chromium trioxide for 12 hours at room temperature (Liau 1971). PCV was measured in 10-ml graduated centrifuge tubes in which the cell suspension was centrifuged for 10 minutes. Fresh weight in each centrifuge tube (pre-weighted) was obtained by substracting the weight of each from the weight of the cell mass plus that of the tube. Growth index (GI) on fresh weight basis was calculated according to the following formula (Stoutemyer and Britt 1969):

$$\text{Growth index (GI)} = \frac{FW - IW}{IW}$$

where FW and and IW are final and initial weight, respectively, GI on PCV basis was determined similarly.

6.2.2.2 Growth regulator

Suspension cultures of both the seedling and adult phases were initiated from callus grown in the dark on M5 medium supplemented with either cytokinin (BA, 2ip, kinetin, zeatin), auxin (IBA, NAA, 2,4-D), or both, each at 1.0 mg/L. A subsequent investigation was conducted using similar medium supplemented with BA in combination either with NAA or 2,4-D, each at concentrations of 0, 0.1, 0.5, 1.0, and 5.0 mg/L. There were four replicate flasks for each growth regulator treatment. Cells in the suspension were plated in 100 x 15 petri dishes each containing 20 ml of solid medium of similar composition but with no growth regulators; cell number, and PCV of cell suspension were measured as described in Section 6.2.2.1.

6.2.2.3 Activated charcoal and coconut milk

Cell suspensions of both seedling and adult phases were cultured on M5 medium containing BA and NAA, each at 0.1, 0.5, 1.0, and 5.0 mg/L, either with or without 1% (w/v) activated charcoal (AC). In a subsequent investigation, suspension of the seedling and adult phases was cultured on both M3 and M5 media supplemented with 0.5 mg/L BA, 0.5 mg/L NAA, and filter-sterilized coconut milk at concentrations of 0, 5, and 10% (v/v). In each investigation, there were four replicate flasks in each treatment. Microscopic examinations of cells were made weekly and cell suspension

was plated as in Section 5.2.2.3 after four weeks. No growth determination was made for all suspensions supplemented with AC, while for that supplemented with coconut milk, fresh weight was measured as described in Section 6.2.2.1.

6.3 Results

6.3.1 Callus culture

White to pale yellow friable callus, suitable for use in suspension cultures, was formed after six weeks at the base of seedling shoot tip explants most frequently on M1 medium containing low cytokinin and high auxin. The presence of 0.5 mg/L BA and 2.0 mg/L NAA was most effective for callus initiation and growth in darkness (Table 35). Callus growth from shoot tip explants of the adult phase was much slower than that of seedling phase. It was observed that callus also grew well under light but green and red pigment were produced on the surface of the callus. Compared to those callus cultures grown in the darkness, the callus appeared more compact and less friable, and was less suitable for use in suspension culture.

TABLE 35. Influence of growth regulators on callus formation of stem tip explants of seedling phase Macspur apple

BA (mg/L)	NAA (mg/L)	CFI[a]
0	0	-
0	0.1	-
0	0.5	+
0	1.0	++
0	5.0	++
0.1	0.1	-
0.1	0.5	+
0.1	1.0	++
0.1	2.0	++
0.5	0.5	+
0.5	1.0	+
0.5	2.0	+++
1.0	1.0	+
1.0	2.0	+
2.0	2.0	+

[a] Callus formation index: -, +, ++, and +++ represent no, slight, moderate, and heavy callus formation, respectively.

6.3.2 Cell suspension culture

6.3.2.1 Culture medium

The dark-grown callus tissues of the seedling phase were readily dissociated and cultured in suspension. Cultures grew into a relatively dense suspension which was creamy-white to pale yellow in color. Cytological examinations revealed that the suspension consisted of mostly single free cells and small cell aggregates.

Growth of cells in suspension from the seedling phase on M1 medium was most rapid between two and four weeks after initiation (Fig. 10). Of the three media (M1, SH, UM), M1 was most effective for growth in cell suspension culture (Table 36). Growth on M1 medium was significantly higher when the macro-elements of this medium was reduced by 1/5 (M5) or 1/3 (M3) (Table 37).

6.3.2.2 Growth regulator

The kind and concentrations of growth regulator in M5 medium influenced differently the growth of cell suspension cultures from seedling and adult phase materials (Table 38). Cells in the suspension from the seedling phase grew well on medium with treatment combinations of auxins and BA or 2ip (each at 1.0 mg/L), with the combinations of

Fig. 10. Time course of growth index (●——●) and cell number (○-----○) of seedling phase Macspur suspension culture on M 1 medium.

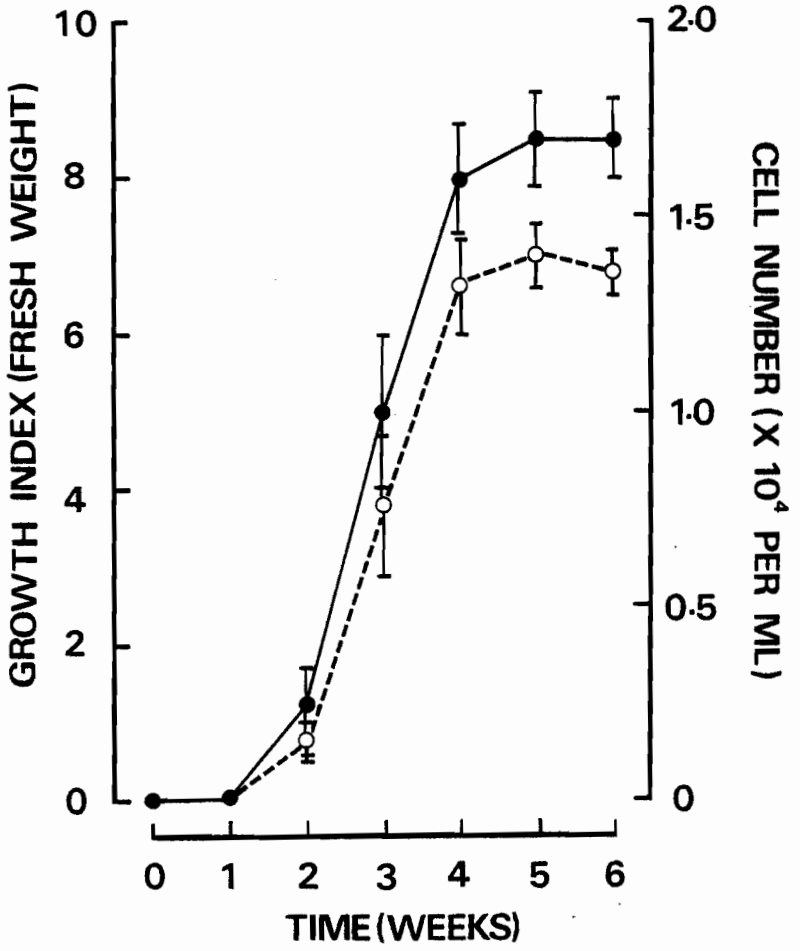

GROWTH INDEX (FRESH WEIGHT)
CELL NUMBER (X 10⁴ PER ML)
TIME (WEEKS)
0
2
4
6
8
10
0
0.5
1.0
1.5
2.0
0
1
2
3
4
5
6

TABLE 36. Growth of seedling phase Macspur cell suspension in different nutrient media

Medium	Cell number ($x10^4$ per ml)	GI[a] Fresh weight (mg)	GI[a] PCV[b] (ml)
Modified Murashige and Skoog (M1)	2.0[c]	6.6	9.0
Shenk and Hildebrandt (SH)	1.2	2.8	6.5
Uchimiya and Murashige (UM)	0.8	1.1	4.0
LSD (p=0.05)	0.6	1.4	2.0

[a] Growth index.

[b] Packed cell volume.

[c] Each datum represents the mean of 10 replications (flasks) each with 25 ml of cell suspension after four weeks in vitro.

TABLE 37. Growth of seedling phase Macspur cell suspension in media with different concentrations of Murashige and Skoog's macro-elements

Medium	Macro-[a] element concentration	Cell number (x10 per ml)	GI[b] Fresh weight (mg)	GI[b] PCV[c] (ml)
M 9	1/9 X	0.9[d]	3.3	7.1
M 7	1/7 X	1.1	4.1	9.2
M 5	1/5 X	3.1	8.7	16.5
M 3	1/3 X	2.9	8.0	11.6
M 2	1/2 X	1.9	6.5	8.8
M 1	1 X	2.2	5.9	8.7
LSD (p=0.05)		0.4	0.8	1.8

[a] MS macro-elements in M 1 medium.

[b] Growth index.

[c] Packed cell volume.

[d] Each datum represents the mean of six replications (flasks) each with 25 ml of cell suspension after four weeks in vitro.

TABLE 38. Influence of growth regulators on growth of seedling and adult phase Macspur cell suspensions

Growth regulator (1.0 mg/L)	Seedling			Adult		
	Cell number (x10^4 per ml)	GI[a] Fresh weight (mg)	GI[a] PCV[b] (ml)	Cell number (x10^4 per ml)	GI Fresh weight (mg)	GI PCV (ml)
Control[c]	0[d]	0	0	0	0	0
BA	0	0	0	0	0	0
2ip	0	0	0	0	0	0
Kinetin	0	0	0	0	0	0
Zeatin	0	0	0	0	0	0
IBA	0.1	0.8	1.2	0.2	1.2	1.4
NAA	0.2	1.2	1.4	0.1	1.2	1.2
2,4-D	0.2	1.1	1.4	0.3	1.8	2.5
BA + IBA	3.0	8.7	15.8	0.8	1.7	2.2
BA + NAA	3.5	9.2	18.4	1.5	4.9	8.5
BA + 2,4-D	2.4	7.3	13.0	1.7	5.6	9.1
2ip + IBA	2.7	8.0	15.4	0	0	0
2ip + NAA	2.3	6.7	11.9	0	0	0
2ip + 2,4-D	1.8	5.4	10.1	0.8	2.9	4.7
Kinetin + IBA	0.2	1.2	1.5	1.2	4.0	6.9
Kinetin + NAA	0.2	1.3	1.7	1.6	5.2	8.8
Kinetin + 2,4-D	0.4	2.0	2.8	2.5	7.4	13.3
Zeatin + IBA	0.1	0.9	1.3	0	0	0
Zeatin + NAA	0.1	1.0	1.2	0	0	0
Zeatin + 2,4-D	0	0	0	0	0	0
LSD (p=0.05)	0.4	0.9	1.3	0.3	0.5	1.0

[a] Growth index.

[b] Packed cell volume.

[c] No growth regulator.

[d] Each datum represents the mean of four replications (flasks) each with 25 ml cell suspension after four weeks in vitro.

1.0 mg/L BA and 1.0 mg/L NAA being the best. On the other hand, the combinaton of kinetin and 2,4-D each at 1.0 mg/L tended to be the best for growth of cell suspension of the adult phase (Table 38). In addition, cells in suspension of the seedling and adult phases also grew well at the concentration combination of 0.5 + 0.5 mg/L BA + NAA and 0.5 + 0.5 kinetin + 2,4-D, respectively (Table 39).

Cytological examinations showed striking differences in cell appearance between the seedling and adult phases. Cells from the seedling phase were spherical to oval in shape, less vacuolated, and the cell wall and protoplasm could be readily distinguished; cells also appeared to be somewhat heterogeneous comprising of two types of cells: cytoplasmic cells (CC) which were small, rich in cytoplasm; cells which were vacuolated (VC) (Fig. 11A). In constrast, cells from the adult phase appeared elongated and highly vacuolated, and the cell wall and protoplasm were indistinguishable (Fig. 11B). In the seedling phase, cells particularly in the medium containing 0.5 + 0.5 mg/L BA + NAA, cultures at the two-celled stage were frequently observed (Fig. 12A), and these at the four-celled stage only occasionally (Fig. 12B). Cell division beyond these stages was not observed. After suspensions were plated, cells from the seedling but not the adult phase formed callus on the medium.

Cell suspension became darkened in medium supplemented with activated charcoal (AC). Growth of these cells was not determined due to difficulty in separating the AC from the cell suspension. Cultures of two-celled and four-celled stages again were observed in the suspension of seedling phase material in the presence of BA + NAA at concentrations

TABLE 39. Influence of different combinations of growth regulator on growth of seedling and adult phase Macspur cell suspensions

Growth regulator	Seedling			Adult		
	Cell number ($x10^4$ per ml)	GI[a] Fresh weight (mg)	GI[a] PCV[b] (ml)	Cell number ($x10^4$ per ml)	GI Fresh weight (mg)	GI PCV (ml)
Kinetin (mg/L) + 2,4-D (mg/L)						
0 + 0	0[c]	0	0	0	0	0
0.1 + 0.1	0.4	2.0	3.0	1.4	3.9	7.9
0.5 + 0.5	0.8	3.1	4.8	2.9	8.2	15.4
1.0 + 1.0	0.5	2.3	3.5	2.5	7.3	13.5
5.0 + 5.0	0	0	0	0	0	0
LSD (p=0.05)	0.2	0.6	0.7	0.7	2.1	2.8
BA (mg/L) + NAA (mg/L)						
0 + 0	0	0	0	0	0	0
0.1 + 0.1	1.4	4.6	8.2	0.8	3.0	4.5
0.5 + 0.5	3.2	7.8	17.0	2.1	6.8	11.8
1.0 + 1.0	2.7	6.9	16.9	1.7	5.2	7.7
5.0 + 5.0	0	0	0	0	0	0
LSD (p=0.05)	1.0	2.3	4.5	0.5	1.6	2.3

[a]Growth index.

[b]Packed cell volume.

[c]Each datum represents the mean of four replications each with 25 ml of cell suspension after four weeks in vitro.

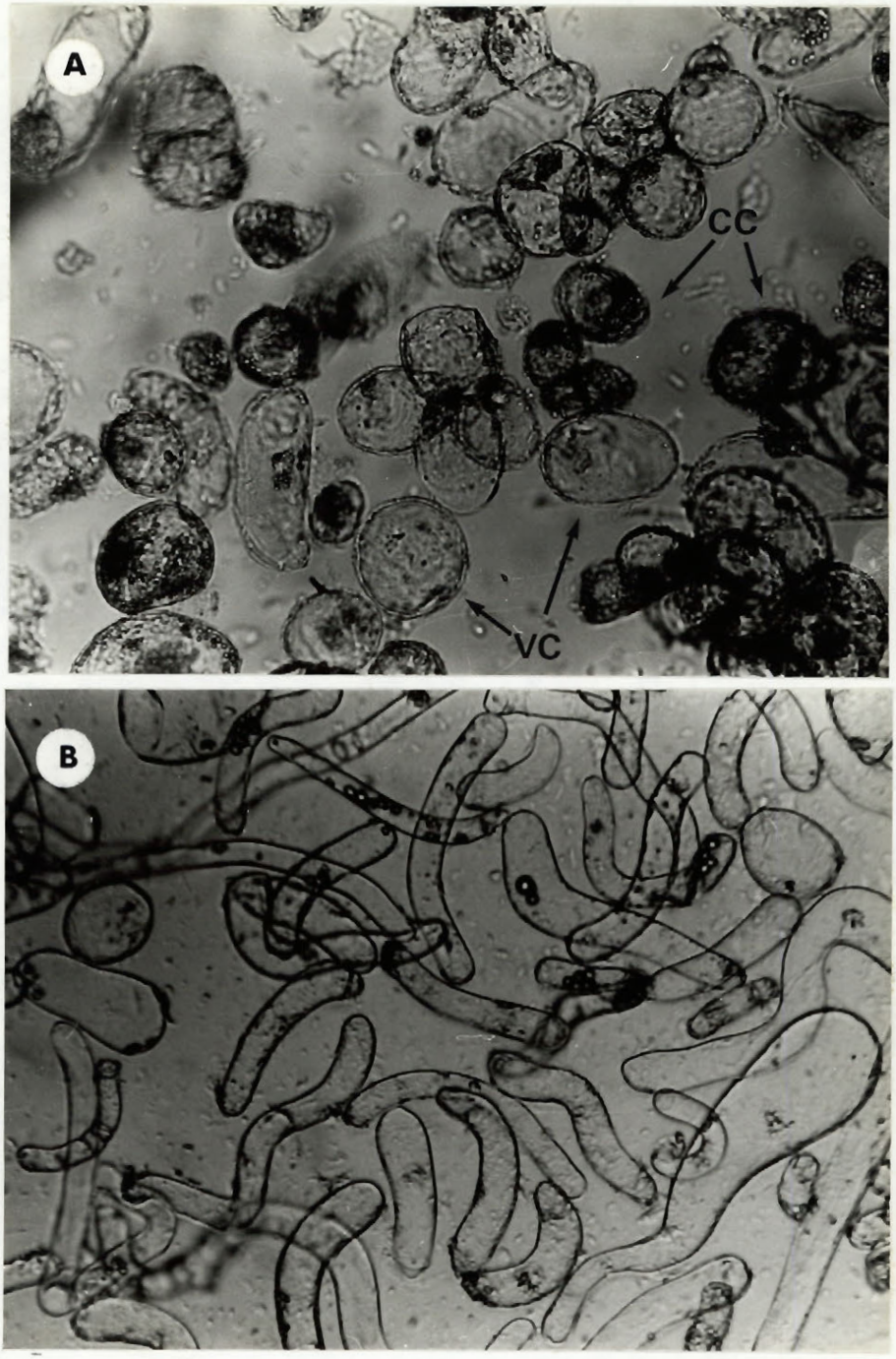


Fig. 11. Comparison of cells in suspension derived from seedling (A) and adult (B) phase Macspur apple. CC, cytoplasmic cell; VC, vacuolated cell. x200.

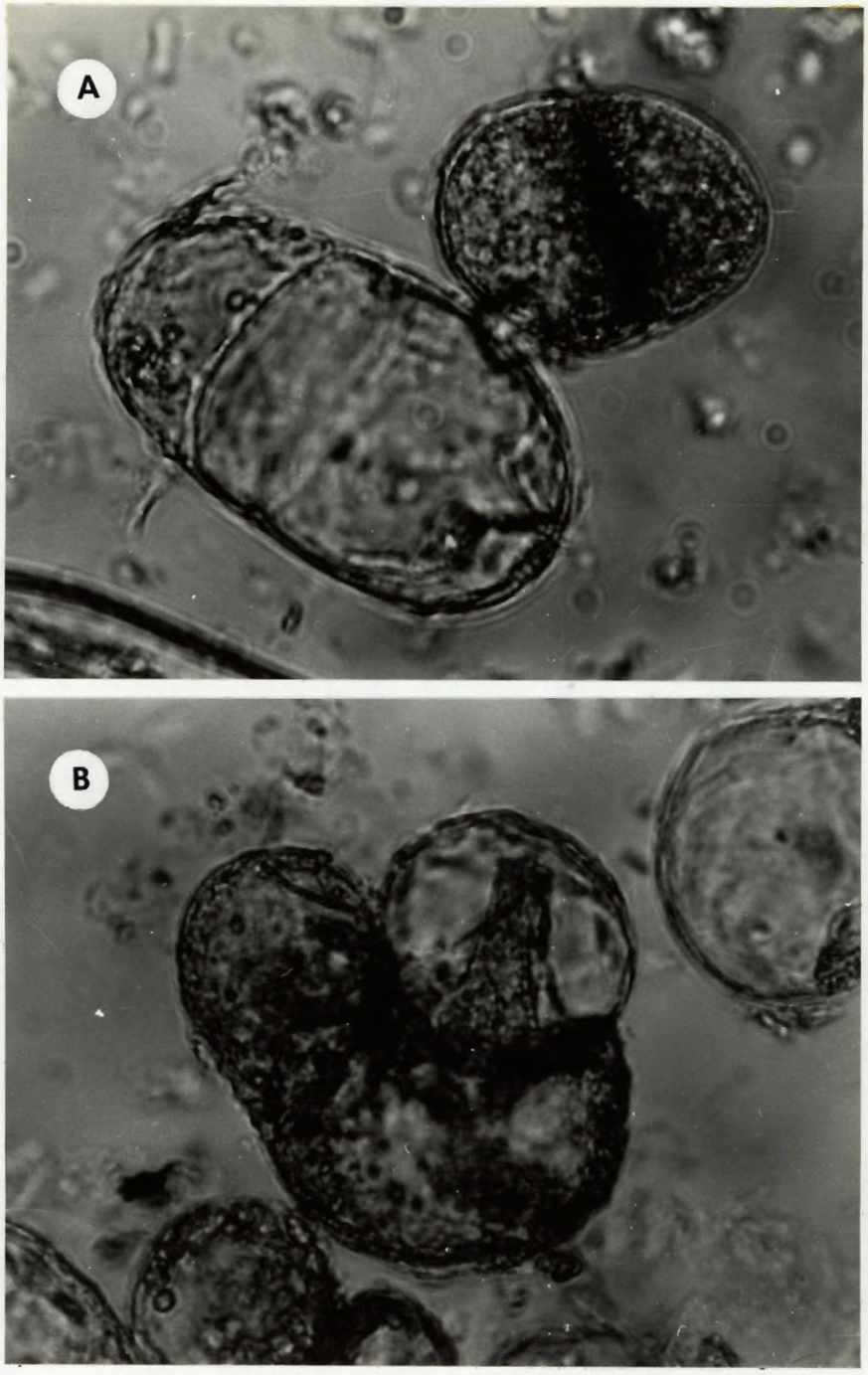


Fig. 12. Two-celled (A) and four-celled (B) stages in suspension culture of seedling phase Macspur apple. x800.

of 0.1 + 0.1 mg/L, 0.5 + 0.5 mg/L with or without AC, or 1.0 + 1.0 mg/L with AC. Later stages of cell division was not observed either in liquid or on solid medium, although callus formation occurred after cells were plated on solid medium.

The presence of coconut milk in both M3 and M5 media resulted in increasing growth but promoted cell aggregation and pigmentation in the suspension. Large cell clumps were observed more frequently in 10% coconut milk than in media with 5% or no coconut milk. More green pigment formed particularly on the surface of large cell clumps in the presence of coconut milk. In addition, fresh weight growth index of cell suspensions in both M3 and M5 media were higher in the seedling than the adult phase (Table 40). However, embryogenesis was not observed either in liquid or on solid media.

6.4 Discussion and conclusions

Cells capable of undergoing embryogenesis can be recognized by a relatively small, isodiametric shape, dense cytoplasm, least vacuolated, prominent nuclei, thin-walled, and conspicuous starch content (Sharp et al. 1980; Vasil and Vasil 1980). Although induction of somatic embryogenesis was not successful in this study, cells from the juvenile (seedling) phase appeared to be more embryogenic competent particularly with regards to the occurrence of cytoplasmic cells (CC) and the fact that cultures of two- and four-celled stages were frequently observed in

TABLE 40. Influence of coconut milk on fresh weight growth index of seedling and adult phase Macspur cell suspensions in M 3 and M 5 media

Growth phase	Coconut milk (%)	GI[a]	
		M 3	M 5
Seedling	0	6.8[b]	7.5
	5	10.3	14.5
	10	12.7	18.9
	Mean	9.9	13.6
Adult	0	0	6.0
	5	8.4	12.3
	10	10.5	18.6
	Mean	6.3	12.3
	LSD (p=0.05)	2.7	1.0

[a] Growth index on fresh weight basis.

[b] Each datum represents the mean of four replications (flasks) each with 25 ml of cell suspension after four weeks *in vitro*.

juvenile phase suspension culture. However, whether or not these two- and four-celled cultures were derived from cell division of single free cells or from dissociation of cell aggregates is not known. The occurrence of these multicellular cultures seems to imply that cells of the juvenile phase have undergone dedifferentiation. Steward et al. (1958) indicated that all parenchyma cells could not directly recapitulate the embryogenic events without passing through the process of dedifferentiation. The concept of cell dedifferentiation in somatic embryogenesis has been widely accepted (Gautheret 1966; Halperin 1970; Thorpe 1980). However, even if cells are dedifferentiated, somatic embryogenesis involves multi-step processes in which each step leads to the establishment of a particular gene activation, allowing transition to the next development state (Street 1978); the development can be arrested at any step in this process.

Somatic embryogenesis in vitro is generally influenced by components of the culture media among which growth regulators are by far the most important. Auxin alone or in combination with cytokinin has been crucial for development of the embryogenic cells in the primary culture (Thorpe 1982). Evans et al. (1981) found that 2,4-D and NAA were the most commonly required auxins; approximately 50% of the crop species required relatively low cytokinins in the primary culture for successful induction of somatic embryogenesis. In the present study, the combination of low cytokinin (0.5 mg/L BA) and high auxin (2.0 mg/L NAA) concentrations was most effective for growth of callus cultures, but induction of embryogenesis either on solid and liquid media was not successful. This indicates that cells undergo mitosis and cytokinesis may not in itself ensure that embryogenesis will occur.

The addition of activated charcoal (AC) has been shown to be required for induction of somatic embryogenesis in certain plant species (Fridborg and Eriksson 1975; Litz and Conover 1980). The beneficial effect of AC is primarily attributed to its ability to absorb excessive growth promoting substances (Constantin et al. 1977; Weatherhead et al. 1978) and/or growth inhibitors (Weatherhead et al. 1978). On the basis of cultures at two- and four-celled stages observed in this study, AC appeared beneficial to cultures supplied with relatively high content of cytokinin and auxin (each at 1.0 mg/L) but inhibitory to those supplied with low content of growth regulators (0.1 mg/L). However, the presence of AC did not seem to influence somatic embryogenesis in these cell suspension cultures.

Some authors claimed that coconut milk is a prerequisite for induction of embryogenic competent cells in callus cultures (Litz and Conover 1982), or for embryo formation in suspension culture (Steward et al. 1964). Coconut milk has been commonly used as an organic supplement in cell suspension cultures from which somatic embryogenesis was induced (Lu and Vasil 1981; Vasil and Vasil 1981). However, no embryogenesis in apple cell suspension culture occurred in the presence of coconut milk even though growth in terms of fresh weight was increased significantly. Increased growth may be due to some cell division factors present in coconut milk including diphenylurea (Shantz and Steward 1952), 9-β-D-ribofuranosylzeatin (Letham 1974), and zeatinriboside (Van Staden and Drewes 1974).

The fact that apple cells in these studies failed to undergo

embryogenesis in vitro may be related primarily with inappropriate culture media and/or explant source also reflects the relative difficulty in obtaining embryogenesis of woody in comparison with herbaceous species. Investigation on caraway indicated that a balance between ABA, GA_3, and zeatin was important for normal embryogenesis (Ammirato 1977). In grapevine, embryo development required GA_3 and 2ip (Mullins and Srinivasan 1976). It appears that, in addition to auxin and cytokinin, other growth regulators may also play an important role in somatic embryogenesis of apple cells. Therefore, it is suggested that growth regulators particularly GA_3 should be included along with auxin and cytokinin in future studies.

In addition to growth regulators, nitrogen source is also crucial in somatic embryogenesis induction, although its importance is primarily exemplified from investigations of carrot cell suspension cultures. Among nitrogen sources, ammonium was most efficient in inducing somatic embryogenesis, while nitrate as a sole nitrogen source was inhibitory (Halperin 1966; Wetherell and Dougall 1976), although ammonium could be replaced sometimes by nitrate (Reinert 1968; Tazawa and Reinert 1969). MS nutrient medium is characterized by high salts in which nitrate is much higher than ammonium. This may be one of the contributory factors which resulted in failure in embryogenesis of the apple cell suspension cultures, since cell cultures in this study were grown exclusively on the medium consisting of MS macro- and micro-elements. However, further clarification is needed in future studies.

The literature suggests that explant source may play an important

role in somatic embryogenesis in vitro. A relatively high frequency of embryogenesis is often derived from cultures of reproductive tissues such as zygotic embryos, ovular and nucellar tissues (Mott 1981; Tisserat et al. 1979); cells from these tissues are pre-embryogenic determined, and their exposure to cultural environments acts primarily to enhance or repress the embryogenic process (Tisserat et al. 1979). Since induction of somatic embryogenesis in apple as well as other woody plants is rather difficult, using tissues from reproductive tissues as the starting materials is probably more promising than those from vegetative tissues.

In addition to reproductive tissues, cultures derived from juvenile source showed more propensity toward embryogenesis (Tisserat et al. 1979) or morphogenesis (Abbott 1978; Tran Thanh Van 1981) than those from adult source. Evidence indicates that juvenile tissues required less growth regulators for growth (Abbott and Whiteley 1976); adult tissues also contained root inhibitors which were not detected in the juvenile tissues (Crow et al. 1971; Paton et al. 1970). These indications suggest that the lack of growth inhibitor(s) and/or the presence of higher concentrations of growth promotor(s) in the juvenile tissues perhaps accounted for the higher potential for embryogenesis or morphogenesis in cultures derived from these tissues in comparison with those from adult tissues.

Evidence of the repressive factors which inhibit embryogenesis is provided by Esan (1973) who demonstrated that these factors were transmittable through grafting, and the removal of these factors induced embryogenesis. The repressive factors were later identified as some

volatile and non-volatile compounds possibly including ABA, auxin, ethylene, GA, and ethanol (Tisserat and Murashige 1977). These indications seem to suggest that the balance between growth promotors and inhibitors in cultured cells or tissues may play an important role in embryogenesis. Furthermore, in comparison with herbaceous species (mostly annuals), the relative levels of success in inducing in vitro somatic embryogenesis in woody perennials, is perhaps attributable, at least in part, to the accumulation of the growth inhibitors in plant tissues especially as the plant grows older.

Very often only some of the cells in the primary culture would dedifferentiate and become embryogenically determined (Street 1979). It is of importance to identify these embryogenically determined cells to gain a better understanding of the early determinative events of embryogenesis. In coffee, embryogenic determined cells could be distinguished from other types of cell in tissue culture via histological examination (Sondahl et al. 1979) but were indistinguishable in carrot tissue culture (Street and Withers 1974). In view of the fact that cells could be identical cytologically but distinct physiologically (Thorpe 1980), it is necessary to develop a more reliable means to differentiate between embryogenic and non-embryogenic determined cells.

Culture media not only provide nutrients for growth and development of cultured cells and tissues in vitro but also act somewhat as mutagens since deviant cells are commonly detected in callus and cell suspension cultures. To date mechanisms of somaclonal variation remain unclear (Larkin and Scowcroft 1981). It appears that rapid clonal

propagation via somatic embryogenesis may not be practically implemented commercially unless mechanisms of cytodifferentiation are better understood and subsequently lead to an efficient control in chromosomal abberations (Krikorian 1982). However, evidence indicates that chromosomal abberations, especially aneuploidy and polyploidy, readily occurred in callus cultures particularly when cultures were prolonged (Bayliss 1980; D'Amato et al. 1980; Narayanaswamy 1977). Using plant cells which undergo direct embryogenesis without passing through intermediate callus state probably can considerably reduce the frequency of deviant cells arising from the cultures. In addition, shortening the culture period and judicious use of a culture medium also may be of importance.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

This study clearly shows that Ottawa 3 and M. robusta rootstock, and Macspur apple can be rapidly propagated through in vitro meristem tip cultures via proliferation of axillary shoots, followed by rooting and acclimatization of the cultured shoots from culture tubes to the greenhouse. Although cytological examinations were not conducted to determine the genetic makeup of the in vitro cultured plants, genetically deviant plants are normally absent in cultures multiplied through axillary shoot proliferation (Murashige 1974). Throughout the present study, each apple culture was homogeneous under these laboratory conditions.

With micropropagation techniques, millions of plantlets can be produced annually in a relatively small space (Murashige 1978; Vasil and Vasil 1980). Although tissue culture plantlets must be grown on in a greenhouse or field until they become marketable, nevertheless, the

astonishingly large numbers should offset this disavantage. The development of a new tissue culture technique for production of Ottawa 3 should result in greater production and greater availability of this rootstock for replacing orchards such as in Quebec and other regions which have suffered from winter injury losses in recent years.

Apple genotypes differ significantly in nutritional and hormonal requirements during shoot proliferation and rooting in vitro (Lane and McDougald 1982; Nemeth 1981). Although carbohydrates are of prime importance for in vitro organogenesis, a high energy-requiring process (Thorpe 1980), there is no previous report on the comparative influence of carbon sources during different stages of the in vitro propagation process.

Gleddie et al. (1983) indicated that the sugars, sucrose, glucose, and fructose, which supported callus proliferation on eggplant (Solanum melongena L.) leaf explants, were also those which supported embryogenesis. This evidence together with knowledge of the unique role of sorbitol in the carbohydrate metabolism of apple and related species as major translocate and metabolite of photosynthesis, and as intermediate metabolite, respiratory substrate, and storage compound in vivo (Chong and Taper 1972), and its known effectiveness as carbon source for in vitro growth of cells (Pech et al. 1974) and callus of apple (Chong and Taper 1972; 1974a; Coffin et al. 1976; Pieniazek et al. 1978) and related species (Coffin et al. 1976), indicate that it may also play a role in in vitro organogenesis. In fact, the results of the present study support this view, and further demonstrate a differential requirement for sorbitol

and related carbon sources during different stages of in vitro propagation of Ottawa 3 and M. robusta rootstocks, and Macspur apple from both juvenile and adult sources.

The importance of sorbitol has been demonstrated in M. robusta in this study (CHAPTER IV). Furthermore, the marked increase in shoot multiplication rate and rooting ability in adult phase Macspur cultures grown on sorbitol medium further demonstrates an important function of sorbitol in in vitro organogenesis of apple. That is, in addition to providing energy in plant tissue cultures (Thorpe 1980), sorbitol is also associated with growth stimulation in vitro which can not be obtained using other carbon sources. However, this effect was genotype dependent. This is shown in Ottawa 3 cultures for which sucrose was the prerequisite carbon source for normal growth. As indicated previously, many apple cultivars (Jones et al. 1979; Lane 1978) and rootstocks (Nemeth 1981; Werner and Boe 1980) propagated in vitro have been grown on sucrose medium.

Since sorbitol and related sugars are normally readily interconvertible (Bieleski 1982), differences in growth and proliferation associated with the utilization of sorbitol and the hexose during the different stages of in vitro propagation may be associated with the availability or lack of one or more of the enzymes (Coffin et al. 1976), such as sorbitol dehydrogenase (Negm and Loescher 1979; 1981), sorbitol-6-phosphate dehydrogenase (Yamaki 1981), and sorbitol oxidase (Yamaki 1982). Response to sucrose may be related to availability of invertase (Chong and Taper 1974a; Maretzki et al. 1974). According to

Thorpe (1978), initiation of organized development involves a shift in metabolism in which new enzymes originally absent are synthesized, or enzymes present show increased synthesis.

The fate of exogenously applied sorbitol and other carbon sources is unknown after uptake by the cultured shoots. This area is worthy of further exploration. This can be accomplished by radioactive tracer study in which the apple cultures are fed with labelled carbon sources, and/or enzymological studies in which the enzymes involved in sorbitol and related carbohydrate metabolism can be isolated and identified.

In addition to carbon sources, growth and morphogenesis of in vitro cultured plant tissues are greatly influenced by other components of the culture medium among which growth regulators have long been recognized (Skoog and Miller 1957). To date there is still no information on changes in endogenous growth regulators during organogenesis (Thorpe 1980). In apple shoot proliferation cultures, the medium is usually supplemented with cytokinin alone (Lane 1980) or in combination with auxin (James and Thurbon 1981) and/or GA (Zimmerman and Broome 1981). The importance of cytokinin and auxin combinations in shoot proliferation of apple cultures has been demonstrated (James and Thurbon 1981), but GA has not been proven to be mandatory in apple cultures. This study demonstrates that GA_3 is a prerequisite for normal growth of the Ottawa 3 cultures but not for cultures of M. robusta or Macspur apple. The exact role of GA_3 in organogenesis is not clear although some observations have favored the hypothesis that GA induces induces biosynthesis of IAA (Coleman and Greyson 1977). The mechanism of interactions between GA and

other growth regulators within the plant also remains to be determined.

Rooting of in vitro cultured shoot cuttings always can be achieved by using the standard nutrient medium supplemented with auxins such as IBA and NAA. In this study, IBA appears to be the most effective auxin for root induction of all apple cultures tested. The naturally occurring auxin, IAA, was not tested in this study since IAA has been shown to be not as effective as IBA (Jones and Hatfield 1976) and NAA (Hartmann and Kester 1983), due presumably to the ease of destruction of IAA by IAA oxidase within the plant (Hackett 1970). In response to auxin treatment, root initials of cuttings were usually induced at the shoot base in stem tissues near or just outside the phloem, in the phloem ray, or in the interfascicular regions between the vascular bundle (Tukey 1979). Although studies indicated that rooting of apple shoot cuttings could not be enhanced by increasing auxin concentration alone (Delargy and Wright 1979), results of this and other studies (Cheng 1978; Zimmerman and Broome 1981) showed that rooting of apple cultures in vitro could be obtained by manipulating the auxin concentration. For difficult-to-root apple cultures such as Ottawa 3, relatively high auxin concentration (6.25 mg/L IBA) is required. Chong (1981) also reported the beneficial effect of using high auxin concentration for rooting some woody species.

Differences in rootability of apple in vitro may be attributed to the inconsistency of media components other than auxins. It is noted that the level of mineral salts is the most common variable among rooting media of apple. Full-strength MS mineral salts were used by Jones and Hopgood (1977) and Jones et al. (1979). Welander and Huntrieser (1981) used

half-strength MS macro-elements, while in this study both MS macro- and micro-elements were reduced by half. According to preliminary investigations in this laboratory, rooting of Macspur apple was markedly improved by using half-strength MS mineral salts. Investigations on the influence of mineral salts on rooting of rose also showed that lowering the total MS mineral salt level resulted in promoting rhizogenesis, particularly the number and length of roots per explant due to a reduction in the nitrogen concentration in the medium (Hyndman et al. 1982a).

In woody plants, ample evidence indicates that tissue cultures derived from adult plants are more difficult to initiate and to establish than those from juvenile plants (Lyrene 1978; 1980). This appeared to be true for M. robusta and Ottawa 3. However, in Macspur, both juvenile and adult cultures were relatively easy to initiate and establish.

It is noteworhty that low shoot proliferation rate in the adult cultures was the common characteristic among apple cultures tested in comparison with the juvenile cultures. Differences between the juvenile and adult cultures also can be detected at the cellular level; cells of juvenile Macspur cultures were less vacuolated and less elongated than those of the adult cultures. Cytological differences between the juvenile and adult tissue cultures also were observed in English and Algerian ivy (Stoutemyer and Britt 1965). Therefore, further research on juvenility at the cellular or even subcellular levels appears to be promising.

Evidence to account for phase changes in woody plants is limited, although some success has been achieved through studies of rejuvenation

of English ivy via application of exogenously applied GA_3 (Rogler and Hackett 1975; Robbins 1957; 1960) or via determination of GA content in shoot apices (Frydman and Wareing 1973a; 1973b). Results of this study indicate that phase change in apple probably involves changes in carbohydrate metabolism since the adult phase Macspur cultures grown on sorbitol medium exhibited a significantly greater vegetative regeneration ability in term of shoot multiplication and rooting. According to Hartmann and Kester (1983), the greater vegetative regeneration ability is one of the important indicators of juvenility.

While many woody plants as well as herbaceous plants are propagated in vitro through shoot tip or meristem cultures, much effort has been devoted to cloning plants via somatic embryogenesis in cell suspension cultures since the first success in regeneration of plants from cells in suspension of carrot was reported by Steward et al. (1958). After more than two decades of trial-and-error, plant species capable of undergoing somatic embryogenesis in cell suspension cultures are far more limited than other methods of aseptic culture for clonal propagation. This is presumably due to shortcomings in understanding of the control mechanism of in vitro embryogenesis (Krikorian 1982; Steward 1970). Before apple, or many other woody plants, can be raised for routine horticultural or agricultural purposes via somatic embryogenesis in cell suspension cultures, more investigations which will contribute to our understanding of the phenomenon of somatic embryogenesis are needed, particularly those at the cellular and molecular levels pertaining to the determination of embryogenic cells and the induction of embryogenic development.

CHAPTER VIII

SUMMARY

1. Using plant tissue culture techniques, Ottawa 3 and Malus robusta No. 5 rootstocks, and Macspur apple derived from seedling and adult phases were successfully propagated through the process of in vitro initiation of meristem tip cultures, shoot multiplication, and root induction of cultured shoots. Furthermore, the influence of carbon sources on the regulation of shoot proliferation and rooting of Ottawa 3 and M. robusta cultures, and juvenile and adult phase Macspur cultures were investigated. Cell suspension cultures were initiated from the two growth phases of Macspur; induction of somatic embryogenesis was attempted from these cultures.

2. For Ottawa 3, the presence of BA and NAA both at concentrations of either 0.5 or 1.0 mg/L in the culture medium was most effective for in vitro shoot proliferation, but growth was abnormal. Normal growth

was achieved when shoots were cultured with a combination of BA, NAA or IBA, and GA_3 on which shoot multiplication rate of four-fold was obtained monthly. Maximum rooting (100%) was achieved after two weeks on agar medium supplemented with 6.25 mg/L IBA.

3. Investigations of carbon source effect showed that shoot proliferation of Ottawa 3 cultures was equally effective on sorbitol, sucrose, and glucose media, but normal cultures were obtained only on sucrose medium at a concentration of 30 g/L. Significantly higher leaf fresh weight and lower stem fresh weight resulted in higher leaf/stem ratio for cultures on sucrose medium in comparison with the other carbon source media. Cultures grown on sorbitol and glucose media were more compact than those on sucrose and fructose media. In response to different sorbitol and sucrose combinations (30 g/L total), increasing proportions of sorbitol and decreasing proportions of sucrose resulted in significantly decreased shoot length, LNI, and CI of Ottawa 3 cultures. For rooting, sucrose at concentrations between 10 and 50 g/L was most effective.

4. For M. robusta, meristem tip explants were successfully initiated in vitro on nutrient medium with sorbitol but not with glucose, sucrose or fructose, each supplied at 30 g/L. Cultures, subsequently subcultured and multiplied on sorbitol medium, produced the highest number of shoots and had the highest shoot length and shoot fresh weight, followed in descending order by cultures grown on glucose, sucrose, and fructose media. Increasing the proportion of sorbitol and decreasing that of sucrose in the carbon source fraction resulted

in increasing shoot number and shoot fresh weight accompanied by decreasing leaf/stem ratio and CI of the shoot cultures. Eighty-five percent rooting was achieved after 32 days on agar medium with 0.5 mg/L IBA in the presence of either sorbitol or sucrose.

5. In seedling phase Macspur, investigations of carbon source effect showed that sorbitol and sucrose were most effective for shoot proliferation. Cultures exhibited significantly higher leaf fresh weight on sucrose medium and lower stem fresh weight on glucose medium which resulted in higher leaf/stem ratio in comparison with those on the other carbon source media. Cultures maintained on sorbitol medium showed significantly higher number of shoots and leaf fresh weight but shorter shoot length and more compactness than those on sucrose medium. Shoot proliferation tended to be best after cultures were grown on sorbitol medium for three generations, then transferred to medium supplied with 25% sorbitol and 75% sucrose (30 g/L total). Maximum rooting was obtained on agar medium supplemented with 0.25 mg/L IBA after three weeks. Both sorbitol and sucrose were the most effective carbon sources for rooting. While cuttings taken from cultures maintained on sucrose medium rooted well in all sorbitol and sucrose combinations, those from cultures on sorbitol medium rooted poorly.

6. In adult phase Macspur cultures, shoot proliferation was slower than seedling phase cultures. The highest number of shoots and stem fresh weight, accompanied with the lowest leaf/stem ratio, occurred on sorbitol medium; growth differences among cultures on the other carbon

source media were less pronounced. For cultures maintained on sorbitol medium for four generations, the number of shoots increased even more but cultures were compact. The best treatment (25% sorbitol + 75% sucrose) was similar to that in the seedling phase. Maximum rooting was obtained on medium with 3.0 mg/L IBA after four weeks. Among carbon sources, sucrose was most effective for rooting. Cuttings taken from cultures maintained on both sorbitol and sucrose media rooted equally well, but root number per rooted cutting was slightly higher for those on sucrose medium.

7. Cell suspension cultures of both seedling and adult phase Macspur were initiated from stem tip-derived callus grown in darkness. In the suspension culture, cells of the seedling phase were spherical to oval in shape, less vacuolated, and the cell wall and protoplasm could be readily distinguished, whereas cells from the adult phase appeared elongated and highly vacuolated, and the cell wall and protoplasm were indistinguishable. While three- to five-fold dilution of the modified MS nutrients were the best basal medium for growth of cells in the suspension, the most effective growth regulators for growth of seedling and adult phase cell suspension cultures were combinations of BA + NAA and kinetin + 2,4-D, respectively, at concentration combinations of 0.5 + 0.5 mg/L and 1.0 + 1.0 mg/L. No embryogenesis was observed in all treatments including the addition of activated charcoal or coconut milk to the culture media; however, cell division up to two- and four-celled stages were detected in seedling phase cell suspension culture.

CHAPTER IX

CONTRIBUTIONS TO KNOWLEDGE

The following contains, to the best knowledge of the author, the original contributions to knowledge that resulted from this research:

1. Methods were developed for obtaining, for the first time, a continuous supply of normal shoot cultures for *in vitro* propagation of Ottawa 3 apple rootstock. In these cultures, gibberellic acid was demonstrated to be mandatory for normal growth of cultures. This results should be of great benefit to apple growers.

2. These research advanced the first report of a differential requirement for sorbitol as carbon source during different stages of *in vitro* propagation of an apple genotype (*Malus robusta* No. 5). The results indicated that for initiation of meristem tip cultures the presence of sorbitol is mandatory; for shoot multiplication the

presence of sorbitol is most beneficial than sucrose, glucose, and fructose; for rooting the presence of either sorbitol or sucrose is beneficial.

3. The finding of this research is of significance in in vitro propagation of apple in view of the fact that shoot multiplication rate of adult phase Macspur cultures maintained on sorbitol medium increased two- to four-fold over standard nutrient medium with sucrose. Furthermore, a remarkable increase in vegetative regeneration ability of adult phase Macspur cultures maintained on sorbitol medium indicates, for the first time, that a partial reversion to juvenility in apple tissue cultures may be related to the presence of sorbitol in the culture medium.

4. Cells in the suspension of the seedling and adult phase Macspur apple were different cytologically and physiologically. This indicates, for the first time, a manifestation of juvenility at the cellular level.

5. This study clearly re-emphasizes the importance of sorbitol as a key carbohydrate metabolite in the apple and, for the first time, demonstrates its regulatory role in in vitro organogenesis for a higher plant. Studies to determine the differential uptake and interconversions of sorbitol and related carbon sources during each stage of in vitro propagation of the apple and related species should elucidate further this role.

CHAPTER X

SUGGESTIONS FOR FUTURE STUDIES

Results obtained in this study suggest some areas that need further investigations:

To further elucidate the regulatory role of sorbitol and related carbohydrates in in vitro organogenesis of apple cultures indicated in the present study, and also to gain a better understanding of the physiology and metabolism of growth control in apple cell and tissue cultures derived from juvenile and adult sources, the following studies are suggested: (1) radioactive tracer studies in which in vitro cultured cells and tissues are fed with labelled carbon sources, followed by extraction and analysis for labelled carbohydrates and their phosphate derivatives; (2) related enzymological studies in which enzymes involved in carbonhydrate metabolism can be determined qualitatively and quantitatively; and (3) determination of the relative contents of

carbohydrates using gas chromatographic techniques.

Furthermore, future investigations should also focus on changes of endogenous growth regulators on in vitro organogenesis of apple cultures since results of this and other studies (Lane and McDougald 1982; Nemeth 1981) showed that growth regulators played an important role in shoot proliferation and rooting of in vitro cultured apple.

For clonal propagation via somatic embryogenesis in cell suspension cultures of apple as well as many woody species, a major effort is needed to investigate the phenomenon of somatic embryogenesis particularly those at the cellular and molecular levels to gain a better understanding of the control mechanism of in vitro embryogenesis. However, evidence obtained from this and other studies (Mott 1981; Tisserat et al. 1979) indicated that somatic embryogenesis was more likely to occur in cultures derived from juvenile phase material or reproductive tissues such as nucellar and ovular tissues. Further investigations on somatic embryogenesis should therefore employ juvenile or reproductive tissues together with manipulation of the culture media components particularly growth regulators such as auxin, cytokinin and gibberellic acid, nitrogen and, perhaps, the carbon source.

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APPENDIX TABLES

APPENDIX TABLE 1. *In vitro* shoot proliferation and rooting of apple

Apple (species and/or cultivar)	Explant source	Stage[a]	Culture medium: Macro[b]	Micro[b]	Vitamin[b] (mg/L)	Sugar (g/L)	Growth regulator (mg/L)	Agar (g/L)	pH	Other[c] (mg/L)	Temp (°C)	Photo-period (hr)	Light intensity (klux)	Reference
Malus domestica Mill.														
A 2	Meristem tip	II	MS	MS	th(0.4)[c]	su(30)[c]	BA(1.0)	7	5.2	mino(100)	24	16	5	Walender and Huntrieser 1981
		III	1/2MS	MS	th(o.4)	su(30)	IBA(2.0)	7	5.2		24	16	5	
Annurca	Shoot tip	II	-[d]	-	-	-	BA(1-2)	-	-	-	-	-	-	Ancora *et al.* 1981
		III	-	-	-	-	NAA(1)	-	-	pg(162)	-	-	-	
Antonovka KA 313	Shoot tip	II	CH	CH	th(2.5)	su(30)	BA(1.13); IBA(0.1-1.0)	0 or 6-10	5.5	mino(250)	-	-	-	Cheng 1978
		III	CH	CH	th(2.5)	su(30)	IBA(0.1-1.0)	6	5.5	mino(250)	-	-	-	
Cortland	Shoot tip	II	MS	DP	-	su(30)	BA(0.1-1.0)	0.75	6	-	25	24	6	Dutcher and Powell 1972
		III	-	-	-	-	-	-	-	-	-	-	-	
Cox's Orange Pippin	Shoot tip	II	LS	LS	th(1.0)	su(20)	Kinetin (0.1-1.0)	7	5.8	mino(100)	25	16	2.6	Abbott and Whiteley 1976
		III	LS	LS	th(1.0)	su(20)	IBA(1.0)	7	5.8	mino(100)	25	16	2.6	
Delicious	Bud	II	MS	MS	th(0.4)	su(30)	BA(2.3)	8	5.7	mino(100); gly(2.0)	23 ± 2 or 26 ± 2	16 or 24	1.5-2.2	Sriskandarajah *et al.* 1982
		III	1/2MS	1/2MS	th(0.2)	su(15)	NAA(1.86) or IAA(17.5)	8	5.7	mino(50); gly(1.0)	26 ± 2	24	6.5	
	Meristem tip	II	MS	MS	th(0.4)	su(20)	BA(1.13)	7	5.3	mino(100); gly(2.0)	28	16	1.5	Lane 1978
		III	1/2MS	1/2MS	th(0.2)	su(20 or lower)	NAA(1.86)	7	5.3	mino(50); gly(1.0)	28	18	0.8	
EMLA-7		See Antonovka KA 313 (Cheng 1978)												
EMLA-9		See Antonovka KA 313 (Cheng 1978)												

(table continued)

APPENDIX TABLE 1. (continued)

Apple (species and/or cultivar)	Explant source	Stage[a]	Culture medium: Macro[b]	Micro[b]	Vitamin[b] (mg/L)	Sugar (g/L)	Growth regulator (mg/L)	Agar (g/L)	pH	Other[c] (mg/L)	Temp (°C)	Photo-period (hr)	Light intensity (klux)	Reference
EMLA-25	Shoot tip	II	MS	MS	th(0.4)[c]	su(30)[c]	BA(1); IBA(0.2)	–[d]	–	–	–	–	–	Cheema and Sharma 1982
		III	–	–	–	–	IBA(2)	–	–	AC(20)	–	–	–	
EMLA-27			See Antonovka KA 313 (Cheng 1978)											
Fuji	Shoot tip	II	MS	MS	th(1)	su(30)	BA(1)	7	–	ino(100)	25	24	2	Ishihara and Katano 1982
		III	MS	MS	th(1)	su(30)	IBA(1)	7	–	ino(100)	25	24	2	
Golden Delicious	Shoot tip	II	MS	MS + FeNaEDTA (20)	th(0.4)	su(30)	BA(1.0); IBA(0.1); GA_3(0.1)	7	5.2	pg(162)	15	16	1.8	Jones et al. 1979
		III	MS	MS + FeNaEDTA (20)	th(0.4)	su(30)	IBA(3); GA_3(0.1)	7	5.2	pg(162)	25	16	–	
Granny Smith	Meristem tip	II	MS	MS	th(0.4)	su(30)	BA(2.3)	8	5.7	mino(100); gly(2.0)	24 ± 2	16	3.6	Sriskandarajah and Mullins 1981
		III	1/2MS	1/2MS	th(0.2)	su(15)	IBA(2.0) or NAA(3)	0	5.7	mino(50); gly(1.0)	26 ± 2	24	0.7	
Griffith	Shoot tip	II	MS	MS	GMO	su(20)	BA(1.1); GA_3(0.34)	8	5.7	–	27	16	4	Huang and Millikan 1980
		III	–	–	–	–	–	–	–	–				
James Grieve			See Golden Delicious (Jones et al. 1979)											
Jonathan	Meristem tip	II	KN + NA_2EDTA	MS	th(0.4)	su(30)	BA(2.0); NAA(0.2)	–	–	mino(100); gly(2.0)	–	–	–	Huth 1978
		III	KN + NA_2EDTA	MS	th(0.4)	su(30)	NAA or IBA (1.0 or 10) GA_3(0.1)	–	–	mino(100); gly(2.0)	–	–	–	
			See Delicious (Sriskandarajah et al. 1982)											

(table continued)

APPENDIX TABLE 1. (continued)

Apple (species and/or cultivar)	Explant source	Stage[a]	Culture medium: Macro[b]	Micro[b]	Vitamin[b] (mg/L)	Sugar (g/L)	Growth regulator (mg/L)	Agar (g/L)	pH	Other[c] (mg/L)	Temp (°C)	Photo-period (hr)	Light intensity (klux)	Reference
M.7	Meristem tip	II	1/2MS	1/2MS	th(0.4)[c]	su(15)[c]	BA(0.5)	5 or 6	5.7	mino(100); gly(2.0)	22	16	6.5	Werner and Boe 1980
		III	1/3MS	1/3MS	th(0.4)	su(15)	IBA(2.0)	2.7	5.7	mino(100); gly(2.0)	22	16	6.5	
	Shoot tip	II	-[d]	-	-	-	-	-	-	-	-	-	-	Jones and Hatfield 1976
		III	KN	MS + FeNaEDTA (20)	-	su(30)	IBA(1.0)	0	5.8	pg(162)	25 ± 2	0	5.7	
M.9	Meristem tip	II	MS	MS	th(0.4)	su(20)	BA(1.13)	7	5.3	mino(100); gly(2.0)	28	16	1.5	Lane and McDougald 1982
		III	1/2MS	1/2MS	th(0.2)	su(20)	NAA(0.19-0.61)	7	5.3	mino(50); gly(1.0)	28	16	0.8	
	Shoot tip	II	LS	LS	LS	su(20)	BA(1-2); IBA(0.1-0.5)	10	5.6	-	25	16	1.8	James and Thurbon 1981
		III	LS	LS	LS	su(20)	IBA(3.0)	10	5.6	pg(16.2-1620)	25	16	1.8	
M.26	Shoot tip	II	DNH	DNH	DNH	su(30)	BA(2.05)	7	5.0	ad(40); glu(50); gly(50); mino(100)	27 ± 1	14	2	Nemeth 1981
		III	-	-	DNH	-	IBA(1.02)	-	-	glu(50); gly(50); mino(100)	27 ± 1	14	2	
	Shoot tip	II	MS	MS + FeNaEDTA (20)	th(0.4)	su(30)	BA(1.0); IBA(0.1); GA_3(0.1)	7	5.2	ino(100); pg(162)	25	16	1.8	Jones et al. 1977
		III	MS	MS + FeNaEDTA (20)	th(0.4)	su(30)	IBA(3); GA_3(0.1)	7	5.2	ino(100); pg(162)	25	16	1.8	
	See M.9 (Lane and McDougald 1982)													

(table continued)

APPENDIX TABLE 1. (continued)

Apple (species and/or cultivar)	Explant source	Stage[a]	Culture medium								Temp (°C)	Photo-period (hr)	Light intensity (klux)	Reference
			Macro[b]	Micro[b]	Vitamin[b] (mg/L)	Sugar (g/L)	Growth regulator (mg/L)	Agar (g/L)	pH	Other[c] (mg/L)				
M.27			See Delicious (Jones et al. 1979); M.9 (Lane and McDougald 1982)											
Mac-9			See Antonovka KA 313 (Cheng 1978)											
Macspur			See M.26 (Lane and McDougald 1982)											
Malling Suntan			See Golden Delicious (Jones et al. 1979)											
McIntosh	Shoot tip	II	LS	LS	th(0.4)[c]	su(30)[c]	Kinetin (1.25); IAA(10)	(pvp,5)[c]	5.6	ino(100); gly(5); CM(150 ml/L)	26	18	-[d]	Walkey 1972
		III	LS	LS	th(0.4)	su(30)	IAA(10)	-	5.6	ino(100)	26	18	-	
MM.104	Shoot tip	II	MS	MS + FeNaEDTA (20)	th(0.4)	su(30)	BA(1); IBA(1); GA_3(0.1)	7	5.8	ino(100)	26	16	2.2	Snir and Erez 1980
		III	MS	MS	th(0.8)	su(20)	IBA(1)	7	5.8	mino(100)	26	16	2.2	
			See M.26 (Nemeth1981)											
MM.106			See MM.104 (Snir and Erez 1980)											
MM.109			See MM.104 (Snir and Erez 1980)											
MM.111			See M.9 (Lane and McDougald 1982)											
Northern Spy	Shoot tip	II	MS	MS	-	su(30)	BA(1); IBA(0.1); GA_3(0.5)	4.8	5.6	-	24 -26	16	2-4	Zimmerman and Broome 1981
		III	1/2MS	1/2MS	-	su(15)	IBA(0.1-1.0)	4.8	5.6	-	24 -26	16	2-4	
Nuggett			See Northern Spy (Zimmerman and Broome 1981)											
Ozark Gold			See Northern Spy (Zimmerman and Broome 1981)											
Spartan			See Northern Spy (Zimmerman and Broome 1981)											
Spuree Rome			See Northern Spy (Zimmerman and Broome 1981)											
Stark Jumbo			See Antonovka KA 313 (Cheng 1978)											

(table continued)

APPENDIX TABLE 1. (continued)

Apple (species and/or cultivar)	Explant source	Stage[a]	Culture medium: Macro[b]	Micro[b]	Vitamin[b] (mg/L)	Sugar (g/L)	Growth regulator (mg/L)	Agar (g/L)	pH	Other[c] (mg/L)	Temp (°C)	Photo-period (hr)	Light intensity (klux)	Reference
Stark Spur			See M.26 (Nemeth 1981)											
Stayman			See Northern Spy (Zimmerman and Broome 1981)											
Summer Jumbo			See Antonovka KA 313 (Cheng 1978)											
M. prunifolia var. ringo 'Marubakaido'			See Fuji (Ishihara and Katano 1982)											
M. X purpurea 'Eleyi'	Shoot tip	II	MS	MS	th(0.4)[c]	su(30)[c]	BA(1 or 2)	6	5.7-5.8	mino(100)	25 ± 1	16	3.4	Singha 1982b
		III	1/2MS	1/2MS	th(0.4)	su(30)	NAA(0.1 or 0.2)	6	5.7-5.8	mino(100)	25 ± 1	16	3.4	
M. siebildii var. zumi 'Calocarpal'			See M. X purpurea 'Eleyi' (Singha 1982b)											
M. sp. 'Almey'			See M. X purpurea 'Eleyi' (Singha 1982b)											
M. sp. 'Dainty'	Shoot tip	II	LS	LS	th(0.4)	su(30)	BA(1.0)	7	5.8	mino(100)	25 ± 2	16	4.4	Norton and Boe 1982
		III	LS	LS	th(0.4)	su(30)	IBA(5.0)	7	5.8	mino(100)	25 ± 2	0	-	
M. sp. 'Golden Hornet'			See M.sp. 'Dainty' (Norton and Boe 1982)											
M. sp. 'Hopa'			See M. X purpurea 'Eleyi'(Singha 1982b)											
M. sp. 'Royalty'			See Antonovka KA 313 (Cheng 1978)											

[a] Stage of in vitro propagation, i.e. II, proliferation; III, rooting.

[b] See APPENDIX TABLE 2.

[c] Abbreviation: AC=activated charcoal; ad=adenine; CM=coconut milk; glu=glutamate; gly=glycine; ino=inositol; mino=myo-inositol; pg=phloroglucinol; pvp=polyvinylpyrrolidone; su=sucrose.

[d] Information is not available

APPENDIX TABLE 2. Various macro- and micro—elements and vitamins of culture media used for in vitro shoot proliferation and rooting of apple

Medium ingredient	Culture medium (mg/L)[a]						
	CH	DNH	DP	GMO	KN	LS	MS
Macro-elements							
Ammonium nitrate (NH_4NO_3)	825	1000	1650	-	-	1650	1650
Ammonium sulfate ($(NH_4)_2SO_4$)	-	-	-	134	500	-	-
Calcium chlorite dihydrate ($CaCl_2.2H_2O$)	220	-	-	150	-	-	-
Calcium nitrate tetrahydrate ($(Ca(NO_3)_2.4H_2O$)	-	260	440	-	1000	440	440
Magnessium sulfate heptahydrate ($MgSO_4.7H_2O$)	185	360	370	250	250	370	370
Potassium chlorite (KCl)	-	65	-	-	-	-	-
Potassium nitrate (KNO_3)	950	1000	1950	2500	-	1900	1900
Potassium phosphate (KH_2PO_4)	85	-	170	-	250	170	170
Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$)	-	165	-	150	-	-	-

(table continued)

APPENDIX TABLE 2. (continued)

Medium ingredient	Culture medium (mg/L)[a]						
	CH	DNH	DP	GMO	KN	LS	MS
Micro-elements							
Boric acid (H_3BO_3)	6.2	3	12	3.0	-	6.2	6.2
Cobalt chlorite hexahydrate ($CoCl_2.6H_2O$)	0.02	0.025	-	0.025	-	0.025	0.025
Cupric sulfate pentahydrate ($CuSO_4.5H_2O$)	0.02	0.025	0.05	0.025	-	0.025	0.025
Ferric phosphate tetrahydrate ($FePO_4.4H_2O$)	-	-	-	-	50	-	-
Ferric sulfate heptahydrate ($FeSO_4.7H_2O$)	6.0	27.8	27.8	27.8	-	27.8	27.8
Manganese sulfate monohydrate ($MnSO_4.H_2O$)	-	5	-	10	-	-	-
Manganese sulfate tetrahydrate ($MnSO_4.4H_2O$)	11.4	-	44	-	-	22.3	22.3
Potassium iodide (KI)	0.8	-	-	0.25	-	0.83	0.83
Sodium ethylenedinitrilo tetraacetate (Na_2EDTA)	7.2	37.3	37.3	37.3	-	37.3	37.3
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.4	0.25	0.5	-	-	0.25	0.25
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	10.6	3	17	2.0	-	8.6	8.6

(table continued)

APPENDIX TABLE 2. (continued)

Medium ingredient	Culture medium (mg/L)[a]						
	CH	DNH	DP	GMO	KN	LS	MS
Vitamins							
Ascorbic acid	-	-	-	-	-	-	-
Calcium pantothenate	-	-	-	-	-	-	-
Nicotinamide	-	-	-	-	-	0.5	-
Nicotinic acid	-	5	-	1	-	0.5	-
Pyridoxin.HCl	-	1	-	1	-	0.5	-
Thiamin.HCl	2.5	10	-	10	-	0.1	0.4

[a] Abbreviation: CH=Cheng; DNH=Dudits, Nemeth, and Haydu; DP=Dutcher and Powell; GMO=Gamborg, Miller, and Ojima; KN=Knudson; LS=Linsmaire and Skoog; MS=Murashige and Skoog.

SHORT TITLE

MICROPROPAGATION OF *MALUS*

ENG-CHONG PUA