National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

FIELD PERFORMANCE AND *IN VITRO* HARDENING STUDIES OF MICROPROPAGATED RED RASPBERRY

by

Ribo Deng

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Plant Science
McGill University, Macdonald Campus
Montreal, Quebec, Canada

October 1992

ISBN 0-315-87943-2

Canada

Suggested short title:

FIELD PERFORMANCE AND IN VITRO HARDENING OF RED RASPBERRY

Foreword

This thesis is submitted in part in the form of original papers suitable for journal publications. Chapters 1 and 2 contain a general introduction and a full literature review of the subject matter. Chapter 3 is a reproduction of a manuscript that is in press in Acta Horticulture. Chapter 4 was submitted to HortScience in November 1992 as a report. Chapter 5 was submitted to Plant Cell, Tissue and Organ Culture in November 1992. Chapter 6 was submitted to the Canadian Journal of Plant Science in September 1992 as a full length paper. Chapter 7 contains the general conclusion and chapter 8 contains a list of the contributions to original knowledge. Chapter 9 contains a full bibliography.

The thesis format has been approved by the Faculty of Graduate Studies and Research of McGill University and follows the conditions outlined in the "Guidelines Concerning Thesis Preparation" described below:

"The candidate has the option, subject to the approval of their Department, of including as part of the thesis the text, or duplicated published text, of an original paper or papers.

- Manuscript-style theses must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation.

- Additional material (procedural and design data as well as description of equipment) must be provided in sufficient detail (eg. in appendices) to allow clear and precise

judgement to be made of the importance and originality of the research reported.

- The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a full overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually in the interest of cohesion.

It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instance, connecting texts are mandatory and supplementary explanatory material is always necessary.

- While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. oral defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear.

Ribo Deng Ph.D. Dissertation

ABSTRACT

FIELD PERFORMANCE AND IN VITRO HARDENING STUDIES OF MICROPROPAGATED RED RASPBERRY

Field performance of micropropagated (MP) and conventionally propagated (CP) red raspberry (*Rubus idaeus* L. cv. Comet and Festival) was examined under hedgerow and stool cane management systems for 3 seasons (1989 to 1991). All MP plants established well compared to 58% survival rates 45 days after planting and 92% survival rates after replanting for CP plants. The MP plants were more vigorous compared with the CP plants for the duration of this study as indicated by more and taller canes. MP 'Festival' in 1990 yielded 2.2 $MT \cdot ha^{-1}$, almost half the yield of established commercial plantings in Quebec, while yields from CP 'Festival' and MP and CP 'Comet' were negligible. The MP 'Festival' crop (8.42 $MT \cdot ha^{-1}$) also outyielded CP 'Festival' (6.8 $MT \cdot ha^{-1}$) and both MP (5.72 $MT \cdot ha^{-1}$) and CP (4.91 $MT \cdot ha^{-1}$) 'Comet' in the second fruiting year. Propagation method had no effects on winter hardiness, photosynthetic capacity nor leaf and stem morphology of either cultivar. The results indicated that MP plants were superior to CP plants for both nursery propagation and fruit production due to their more consistent establishment and increased vigor. Red raspberry plantlets were successfully hardened in vitro on low-sucrose or sucrose-free media through CO_2 enrichment (1500 ppm) and relative humidity reduction (90%) using a forced ventilation system in specially constructed plexiglass chambers. Enriched CO_2 significantly increased general vigor, root formation, root growth, plantlet growth and plantlet photosynthetic capacities. Sucrose in the medium promoted plantlet growth but depressed photosynthesis. In vitro relative humidity at 90% decreased stomatal apertures and improved plantlet ex vitro performance but did not affect the CO_2 uptake rates of cultured plantlets or ex vitro transplants. The

maximum CO_2 uptake rates of plantlet leaves were about 52 - 69% that of greenhouse control plant leaves. In vitro hardening of micropropagated red raspberry has significant commercial implications.

Résumé

Études sur l'endurcissement et la performance au champ des vitroplants de framboisiers

Les performances en champ de framboisiers (*Rubus idaeus* L. cv. Comet et Festival) provenant de vitroplants (VP) et de plants progagés de façon conventionnelle (PC), ont été étudiées sous deux types de systèmes d'exploitation, et ce durant trois saisons consécutives (1989 à 1991). Tous les vitroplants ont survécu à la transplantation en champ. Par contre, seulement 58% des plants PC ont survécu 45 jours après la transplantation, et même après remplacement des plants morts, seul 92% survécurent. Les vitroplants étaient plus vigoureux que les plants PC. Ceci fut reflété par un nombre accru de tiges produites par les vitroplants et par une augmentation de la taile de celles-ci tout au long de cette étude. En 1990, les vitroplants du cultivar "Festival" ont produit 2,2 mt ha^{-1}, soit près de la moitié du rendement des plantations commerciales québécoises. Les plants PC de "Festival" et les plants PC et VP de "Comet" n'ont atteint que des rendements négligeables. Durant la seconde année de production, les vitroplants de "Festival" ont également atteint un rendement supérieur (8,42 mt ha^{-1}) par rapport aux rendements des plants PC de "Festival" (6,8 mt ha^{-1}) et des plants VP (5,72 mt ha^{-1}) et PC (4,91 mt ha^{-1}) de "Comet". La méthode de propagation n'a eu aucune influence sur la rusticité, l'activité photosynthétique, ainsi que sur la morphologie des feuilles et des tiges, et ce pour les deux cultivars. Les résultats indiquent que les VP sont supérieurs aux PC, pour ce qui a trait à la propagation en pépinière et la production fruitière, dû à leur vigueur accrue et leur taux de survie plus élevé. Les vitroplants ont été endurcis avec succès sur des milieux de culture in vitro, sans ou avec peu de sucrose, avec un taux de CO_2 de 1500 ppm, ainsi qu'une réduction du niveau d'humidité relative (90%) grâce à un système de ventilation à air forcé à l'intérieur d'une chambre spéciale en plexiglass.

L'enrichissement de l'atmosphère en CO_2 a augmenté la vigeur des plants, la formation et la croissance des racines, ainsi que la croissance et l'activité photosynthétique des vitroplants de façon significative. Le maintien des plants in vitro à un niveau d'humidité relative de 90% a réduit l'ouverture stomatale et a augmenté la performance ex vitro des vitroplants mais n'a eu aucun effet sur la consommation de CO_2 des vitroplants produits in vitro ou ex vitro. La consommation maximale de CO_2 des feuilles de vitroplants était de 52 à 69% celle des feuilles provenant de plants contrôles propagés en serre. L'endurcissement in vitro de microplants de framboisiers offre des avantages commerciaux significatifs.

Acknowledgements

I would like to express my sincere appreciation to my thesis supervisor Dr. Danielle Donnelly for her consistent and valuable supervision, guidance, encouragement and her financial support during the course of this research. I am also very grateful for her editorial help to my thesis.

I would like to thank Dr. Deborah Buszard, a member of my Ph.D. supervisory committee, for her help in the field trial, in reviewing my manuscripts and her financial contribution to this research.

I am thankful to Dr. Don Smith, a member of my Ph.D. supervisory committee, for his help and providing the portable photosynthesis system.

I would also like to thank Dr. Adam Dale of the Ontario Horticultural Research Institute at Simcoe, Ontario for his valuable discussions and constructive criticisms.

My appreciation is also extended to Drs. Hugh A. Daubeny, Bruce Coulman and N. Barthakur for their reviews of my manuscripts.

The valuable help from the Macdonald orchard field crew; Mr. J.P. Laplaine, head technician, Mr. Mike Bleho and the assistance of summer students Ray Watson and Christal Legoux during the field trial is very much appreciated.

Scholarship funds provided by McGill university in the form of an international student differential fee waiver are gratefully acknowledged.

I wish to thank Dr. Sam Sparace for his help and providing the Beckman LS-5801 liquid scintillation spectrometer and the Du-40 spectrophotometer, and Dr. A. Watson for providing the Leco 2001 image analyzer. I thank Helen Cohen-Rimmer for her help with the photographic aspect of this research.

Special thanks are extended to the students and staff of the Department of Plant Science, especially Johanne Cousineau, Yves Leclerc, Maria Jose de Faria, Martine Korban, Linda Nelson, Linda Champagne, Adolfo Minero, Claret Clavijo, Stephane Bailleul, Pascale Maynard, Aline Grenier, Stewart Leibovitch, May Couture, Cindy Smith and Guy Rimmer for their help and kind cooperation.

I am truly indebted to my young daughter Yan for her understanding and for putting up with her busy father who could not give her all the attention she deserved.

Contribution of co-authors to manuscripts for publication

All manuscripts were co-authored by my supervisor Dr. Danielle Donnelly and myself. One manuscript was coauthored by Drs. Danielle Donnelly, Deborah Buszard and myself. Dr. Danielle Donnelly provided help in the form of doctoral supervision, reviewing and some editing of manuscripts which I had written.

I designed, directed, executed and analyzed the results of all experiments. Macdonald orchard technicians J.P. Laplaine and Mike Bleho, and summer students Ray Watson and Christal Legoux provided some help with the field trial in 1989, 1990 and 1991.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 Introduction

1.1 General introduction

Red raspberry (*Rubus idaeus* L.) is conventionally propagated (CP) vegetatively by suckers (Jennings, 1988; Crandall and Daubeny, 1990). Micropropagation (MP) provides a viable propagation alternative which can be used to produce high quality red raspberry plants considerably more quickly (Donnelly and Daubeny, 1986; McPheeters et al., 1988; Snir, 1988). However, the potential of micropropagation has not been fully exploited for red raspberry due to the lack of long term field performance data and the high costs associated with commercial scale micropropagation. This study included long-term field evaluation of MP red raspberry plants and the development of in vitro hardening procedures for red raspberry plantlets.

Conventional propagation can not always be depended on to produce sufficient planting material to meet market demands, especially for the difficult to propagate, newly introduced or newly released cultivars (Snir, 1981; Swartz and Lindstrom, 1986). This is due to the relatively low propagation rates and to growing season constraints. Potential risks of spreading viruses and other disease-causing organisms, from infected source plants to new plantations (Chamber, 1961), are minimized through the use of certification programs (Jennings,

1988; Ellis et al., 1991).

Micropropagation of red raspberry is increasingly done commercially (Zimmerman and Jones, 1991). Several short term greenhouse and field studies indicated that MP red raspberry behaved differently from CP plants (Neal et al., 1990; Trinka and Pritts, 1990). MP 'Heritage' red raspberry was less tolerant to some herbicides (Simazine and Orysalin), during the first 4 weeks ex vitro but could subsequently be sprayed safely (Neal et al., 1990). Field evaluations of MP blackberry (*Rubus eubatus* spp.) (Swartz et al., 1983), loganberry (a hybrid of *Rubus ursinus* X *Rubus idaeus* cv Thornless Logan) (Rosati et al., 1986), strawberry (*Fragaria* x *ananassa* Duch) (Swartz et al., 1981; Damiano et al., 1983; Marcotrigiano et al., 1984; Grout and Millam, 1985; Cameron et al., 1985, 1989; Cameron and Hancock, 1986; Theiler-Hedtrich and Wolfensberger, 1987), apple (*Malus domestica* Borkh) (Rosati and Gaggioli, 1987; Zimmerman and Steffens, 1989; Zimmerman and Miller, 1991), peach (*Prunus persica* L.) (Rosati and Gaggioli, 1987; Hammerschlag and Scorza, 1991) and cherry (*Prunus cerasus* L.) (Rosati and Gaggioli, 1987) indicated that MP plants were generally more vigorous, more uniform and in many cases yielded more than CP plants. Long term evaluations of MP red raspberry had not been done at the time this research project was initiated but are prerequisite to recommendations regarding the use of micropropagation for commercial nursery propagation and fruit production.

Micropropagated plantlets possess a unique culture-induced phenotype (CIP) which is different from that of greenhouse- or field-grown plants. This phenotype prevents MP plantlets, transferred directly from culture, from growing normally under ambient ex vitro conditions without special handling. MP plantlets must undergo a period of acclimatization during which they slowly adjust to ex vitro greenhouse or field environments. This usually involves exposure to high relative humidity (RH) and relatively low light intensity for several days or weeks followed by a gradual weaning period in which RH and light are adjusted towards ambient conditions. This process can be expensive in terms of labor and controlled environment facilities. In vitro hardening involves manipulation of the culture environment to alter the CIP towards photoautotrophy and away from excessive water loss and could abbreviate or eliminate the ex vitro acclimatization period and reduce the overall costs of micropropagation (Donnelly and Tisdall, 1992).

1.2 Objectives

The objectives of this investigation were to:

1.) Compare the field establishment, winter hardiness, productivity, and photosynthetic activity of MP and CP 'Comet' and 'Festival' red raspberry under hedgerow and stool cane management systems in Quebec, Canada.

2.) Promote in vitro hardening by manipulating the culture microenvironment, including CO_2, relative humidity (RH) and medium sucrose levels and assess the effects on MP red raspberry phenotype both in vitro and ex vitro.

Chapter 2 Literature review

2.1 *Rubus idaeus* L.

The red raspberry belongs to the family Rosaceae, genus *Rubus* and subgenus *Idaeobatus*, and is widely distributed in all temperate regions of Europe, Asia, and North America. The most important species in the genus includes the European (*R. idaeus* subsp. *vulgatus* Arrhen.) and North American (*R. idaeus* subsp. *strigosus* Michx.) red raspberry and the black raspberry (*R. occidentalis* L.). The purple raspberry group (*R.* x *neglectus* Peck) is comprised of hybrids of various red and black raspberry. The European raspberry has glandless inflorescences and thimble-shaped fruit while the American raspberry has glandular inflorescences and round fruit. Raspberry can be distinguished from other *Rubus* species as their mature fruit readily separates from the receptacle (Jennings, 1988).

Red raspberry was first domesticated in Europe and has been cultivated for 400 to 500 years. In North America, all red raspberry cultivated before 1800 were of European origin. Superior cultivars were then derived from hybrids of the European and American red raspberry and became the major commercial cultivars of today.

Red raspberry has biennial shoots that grow on a perennial root system (Hudson, 1959; Jennings, 1988). The

biennial growth cycle of red raspberry canes starts with the elongation of root buds (root suckers) or basal axillary buds (stem suckers). These suckers grow vegetatively throughout the first growing season and are termed primocanes (Bailey, 1941). Flower buds are initiated on primocanes in late summer or fall when primocane growth has slowed down or stopped. Flower initiation is probably triggered by the short day length and the low temperatures in late summer and fall (Williams, 1959c). After a period of dormancy (800 - 1700 hours depending on the cultivar) at low temperatures (<7 C) (Crandall and Daubeny, 1990), fruiting laterals develop from the previous year's primocanes, now termed floricanes (Bailey, 1941). Various numbers of flowers and fruit develop on the axillary buds of the fruiting laterals. In North America bloom starts in early summer. Most commercial red raspberry cultivars are self-fertile. Better fruit set and fruit quality are ensured by insect pollination. The red raspberry fruit mature 30 - 40 days after anthesis. The fruit are aggregates of many druplets; each derived from one of the two ovules present in each pistil. The receptacle remains on the lateral when the fruit is picked at maturity. The floricanes die after fruiting (Jennings, 1988).

Primocane fruiting is the one exception to red raspberry fruiting habit. In some cultivars, fruit are produced at the upper portion of 1-year-old canes at the end of the growing season, while the lower portion of the canes fruit the

following season (Keep, 1961; Ourecky, 1975). One of the early significant primocane fruiting cultivars was 'Lloyd George'. 'Heritage' is one of the current popular primocane fruiting cultivars (Jennings, 1988).

Commercial cultivation of red raspberry is mostly located in the colder temperate regions including parts of North America, Europe and southern New Zealand and Australia (Crandall and Daubeny, 1990). Globally, red raspberry has become an important small fruit crop showing increases in annual production for the last three decades from 107,346 MT in 1961-1965 (FAO, 1975) to 375,777 MT in 1989 (FAO, 1989).

In Canada the annual production of red raspberry has also increased from 7,742 MT in 1961-1965 (FAO, 1975) to 23,694 MT in 1988 (Statistics Canada, 1989). Most of the Canadian production occurred in British Columbia (90%), about 6% was in Quebec and 4% was in Ontario and the Atlantic provinces (Statistics Canada, 1989).

Quebec produced 1,361 MT of red raspberry with a farm gate value of 5 million dollars in 1991 which rose from 640 MT and 1.9 million dollars in 1982 (anonymous, 1992). The red raspberry production area in Quebec also rose from 790 ha in 1982 to 1,047 ha in 1991. However, the average fruit yield rose only from 1.23 $MT \cdot ha^{-1}$ in 1982 to 1.67 $MT \cdot ha^{-1}$ in 1991. The most commonly grown red raspberry cultivars in Quebec in 1991 were (in order of importance): Boyne, Festival, Killarney, Madawaska, Carnival, Gatineau and Newburgh; they accounted for

more than 95% of the total area and production in the province.

2.2 Red raspberry propagation

2.2.1. Conventional propagation

Red raspberry is reproduced sexually by seeds or vegetatively by suckers (Hudson, 1959; Shoemaker, 1978; Jennings, 1988; Crandall and Daubeny, 1990). Propagation by seeds is used only by breeders. Propagation by suckers is the most commonly used method in the nursery. Root suckers are derived from root buds which occur throughout the red raspberry root system, even on the small roots, although there may be more and larger buds on larger roots. Root buds on main roots tend to be more active in producing root suckers than those on small roots (Hudson, 1954; Williams, 1959a). Root suckers are the primary source of conventional propagules (Whitney, 1982). Root buds develop early in the fall and emerge from the soil to become root suckers, usually within a radius of 20 cm from the parent canes (Williams, 1959a). These shoots develop a primary rosette of leaves at the apex and soon become dormant. Those root buds that develop later in the fall, mainly between 20 and 80 cm from the parent canes (Williams, 1959a), may not emerge above the soil level before dormancy occurs. The following spring root suckers develop

into primocanes which soon form their own root systems and can subsequently be separated from the parent plant (Whitney, 1982; Dale 1987).

Stem suckers derive from basal axillary buds of floricanes below the soil level and are similar in development to root suckers. The area of subterranean stem basal nodes where stem suckers originate is sometimes referred to as the replacement zone, because stem suckers growing from this zone replace the mother canes when they die after fruiting (Jennings, 1988). After one year's growth, suckers may be dug with their roots attached, usually after dormancy occurs, and either heeled-in or held in cold storage until planted. The development of suckers is cultivar dependent and is greatly influenced by field management system and environmental conditions (Williams, 1959b; Lawson and Wiseman, 1979).

Red raspberry can also be propagated by root (Hudson, 1954; Torre and Barritt, 1979) or leafy (Howard et al., 1987) cuttings. Root cuttings are usually taken during the fall or winter, cold-stored at about 0C for several months, and planted in the spring. This propagation method may supplement the use of suckers to improve the vegetative propagation rate (Torre and Barritt, 1979). Leafy summer cuttings may be used to avoid soil-borne pathogens that affect suckers and root cuttings (Howard et al., 1987).

2.2.2 Micropropagation

Sometimes, conventional methods can not meet the demand for specific pathogen tested (SPT) planting material, especially of those difficult to propagate, newly introduced or newly released cultivars (Snir, 1981; Swartz and Lindstrom, 1986). Micropropagation provides another means of propagation with minimal risk of spreading pathogens to new plantations and with much greater propagation ratios.

Early attempts to culture red raspberry aseptically in vitro were mainly to obtain virus-free material (Shchelkunova and Popov, 1970; Putz, 1971; Vertesy, 1979). Virus elimination can be accomplished through meristem tip culture (Moore and Janick, 1975; Quak, 1977; Styer and Chin, 1983) and the efficacy is enhanced when used in conjunction with thermotherapy (Pyott and Converse, 1981). Rapid micropropagation of SPT red raspberry has been successful (Anderson, 1980; James et al., 1980; Snir, 1981; Swartz et al., 1983; Welander, 1985; Sobczykiewcz, 1987; Hoepfner, 1989). This subject has been reviewed by Donnelly and Daubeny (1986), Snir (1988), McPheeters et al. (1988) and Zimmerman (1991).

Micropropagation of red raspberry is usually started with the aseptic isolation of shoot (or meristem) tips from SPT greenhouse-grown source plants. Alternatively, SPT in vitro plantlets can be used to begin the propagation cycle. If

mother plants are from the field or greenhouse, surface sterilization is required. The upper parts (20 - 40 cm) of actively growing canes are taken from the field or greenhouse and kept in plastic bags moistened by wet tissue paper. Once in the laboratory, the canes are trimmed of all leaves and petioles except 1 cm of each petiole to protect the buds, then cut into sections 5 - 10 cm long consisting of 1 or 2 nodes. Both apical and axillary buds may be used. Surface sterilization is usually achieved by leaving the cane sections under running tap water for 0.5 to 1 hour before agitating in 10% bleach (0.6% sodium hypochlorite) for 15 - 20 min followed by rinsing 2 - 3 times with sterile water. The isolation procedure is done under aseptic conditions in a laminar air-flow cabinet with the aid of a dissecting microscope. Bud scales and leaf primordia are carefully removed to expose the shoot tips or meristem tips. The shoot tip or meristem tip explants are excised and placed onto culture medium (Stage I; Murashige, 1974).

The isolated explants may be cultured in liquid or on agar-solidified modified MS (Murashige and Skoog, 1962) medium (Donnelly and Daubeny, 1986). Most workers incubate red raspberry cultures under 10 - 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, 16 hour photoperiod at 20 - 26C. The established shoots are usually transferred onto agar-solidified medium for multiplication (Stage II). Shoot proliferation is achieved through axillary (lateral) bud development. Axillary buds are

stimulated to develop on proliferation media and the resultant shoot clusters are then divided into individual axillary shoots. They can be repeatedly subcultured onto fresh medium for further shoot proliferation. The cycle can be repeated until desirable numbers of shoots are produced. A propagation rate of 3 - 26 can be achieved during each 3 - 4 week-subculture-period. When sufficient numbers of shoots have been obtained they can be rooted in vitro in rooting medium (Stage III). The rooting medium is the same as for proliferation except cytokinin is excluded or substantially reduced and auxin is increased 2 - 4 times. They can also be rooted ex vitro in covered flats or mist frames. It may be more economical for some species to root Stage II shoots ex vitro. Cultured shoots or plantlets must undergo a period of ex vitro acclimatization before they can be transferred to ambient greenhouse or field conditions. Ex vitro acclimatization will be discussed in detail later in section 2.5.2. Micropropagation has been successfully accomplished for other *Rubus* species, such as blackberry (Broome and Zimmerman, 1978; Harper, 1978; Babic and Neskovic, 1984; Fernandez and Clark, 1991) and tayberry (a red raspberry X blackberry hybrid) (Harper, 1978).

2.2.3 Red raspberry certification programs

Conventionally, vegetative propagation methods risk

introducing pathogens to new plantations if the source plants are infected with viruses and/or other disease-causing organisms (Chamber, 1961; Jennings, 1988). Such problems are minimized through the use of certification programs (Jennings, 1988; Ellis et al., 1991). A commercial red raspberry field requires replanting every 8 - 10 years due to stand degeneration caused by viruses or other disease causing organisms (Agriculture Canada, 1984). Replanting and industry expansion generate a need for continual supplies of certified planting stock.

The supply of healthy planting stock is usually ensured by the red raspberry certification programs organized and supervised by various government agencies. The red raspberry certification programs are quite similar among various locations in North America. The Canadian red raspberry certification programs in British Columbia (B.C.), Ontario, Quebec and Nova Scotia are the responsibility of the provincial ministries of agriculture. Agriculture Canada provides SPT nuclear stock to the provincial horticultural research institutes where the nuclear stocks are further propagated to produce elite stock in strictly protected screen-houses. Certified red raspberry propagators obtain elite stock from the provincial horticultural research institutes and produce foundation and certified stock in their propagation fields. Usually the propagators use the elite stock to produce foundation stock for 1 or 2 years and then

use the foundation stock to produce certified stock for 1 or 2 additional years. The certified stock (commercial stock) is then offered for sale to commercial fruit growers.

Certified red raspberry propagators must follow the guidelines of the certification programs. The certification guidelines usually include:

1. Isolation of the propagation fields. The propagation fields should be at least 450 m from commercial red raspberry fields and at least 150 m from wild raspberry plants.
2. Soil restriction. The propagation fields should not have been used for growing raspberry, tomato (*Lycopersicum esculentum* L.), potato (*Solanum tuberosum* L.), pepper (*Capsicum frutescens* L.) or eggplant (*Solanum melongena* L.) during the previous 5 years.
3. Soil fumigation. Soil in the fields should be fumigated to control nematodes.
4. Insect control. The propagators should follow the insecticide program approved by the provincial horticultural research institute or the department of agriculture.
5. Cultural practices. The propagators should follow the recommendations devised by the provincial horticultural research institute or the department of agriculture.
6. Tolerance. The red raspberry certification programs demand zero tolerance for cultivar mixing; 0.5% for virus infection and 1% for verticillium wilt (*Verticillium albo-atrum* Reinke & Berthier) but all plants with visible symptoms

and those arising from the same root system must be destroyed; all plants with crown gall (*Agrobacterium tumefaciens* Coon) on roots must be destroyed; nematode populations in soil samples must not exceed 100 nematodes•kg^{-1} of soil for root lesion nematode (*Pratylenchus penetrans* de Man) and must not exceed 10 nematodes•kg^{-1} of soil for dagger nematode (*Xiphinema americanum* Cobb).

7. The provincial ministries of agriculture are responsible for inspection of the certified propagator's propagation fields.

Only red raspberry plants produced vegetatively from foundation stock that meet the required tolerances are recognized as certified stocks and allowed to be sold to red raspberry growers. The red raspberry certification programs are open-ended flush-through systems. After certified stocks are sold to growers they become commercial stock and must no longer remain in or be returned to the certification program.

The B.C. Raspberry Certification Committee which consists of representatives from Agriculture Canada, the B.C. Ministry of Agriculture and Fisheries and the University of British Columbia establish, revise and overlook the implementation of the certification guidelines and carry out field inspection of foundation and certified stock. The B.C. Raspberry Certification guidelines were detailed in B.C. by the Ministry of Agriculture and Fisheries (1987). Red raspberry certification programs have also been discussed by the Quebec

Ministere de l'Agriculture, des Pecheries et de l'Alimentation (1992), Dale and Vanderberg (1989) in Ontario and the Nova Scotia Department of Agriculture and Marketing (1990).

Maintenance and propagation of nuclear, elite and foundation stock are expensive for both certified propagators and the regulatory agencies because they require costly, well-isolated screen-house and greenhouse facilities (Goff, 1986). Nuclear plants are maintained in screen-houses and each plant occupies a large area so only a limited number of nuclear plants can be maintained. Several successive generations of field propagation are needed to produce sufficient foundation and certified stock which inevitably increases the risk of reinfection by the insects and pathogens which compromise plant quality. Swartz and Lindstrom (1986) estimated that at least 5,000 foundation plants were needed to produce one million units in 3 years, which required about 1 acre of screenhouse, costing between $ 100,000 to $ 200,000. In comparison, hundreds of thousands of plants may be produced from a single shoot tip in 1 year through micropropagation and there is negligible chance of reinfection by plant pathogens. For the certified stock, the closer they are to the micropropagated generation the better their quality in terms of health status. Micropropagation has been incorporated into the red raspberry certification program to rapidly propagate SPT nuclear stock. The more prolific growth of MP plants compared with CP plants may be further exploited in later

stages of the certification cycle. Fewer nuclear stock plants would be needed in the maintenance screen-houses which would translate into appreciable savings in maintenance, testing, and inspection costs.

Micropropagation of red raspberry has become commercialized (Swartz and Lindstrom, 1986; Zimmerman, 1988). In 1984, 12,000 (Canada) and 1.2 million (U.S.A.) red raspberry plants were produced by micropropagation which accounted for 1% and 12%, respectively, of total plants produced (Swartz and Lindstrom, 1986). The relatively small number of plants produced by micropropagation was due to high costs. For example, in 1984 the wholesale price for MP plants averaged $0.45 per unit whereas field produced plants averaged $0.35. The current number of MP raspberry plants produced is considerably higher due to wider grower acceptance (Zimmerman, 1991). However, the cost of micropropagation is generally higher than that of conventional methods for a wide range of species (Donnan, 1986) including ornamental (Jones, 1986; Rowe, 1986; Pierik, 1988; Chu and Kurtz, 1990; Sagawa and Kunisaki, 1990) and small fruit and grape (Swartz and Lindstrom, 1986), so the use of micropropagation is mostly limited to the production of nuclear and elite stock. The conventional vegetative propagation methods are still being used extensively to propagate foundation and certified stock. Should the costs of micropropagation decrease, the advantages of this technology could be better exploited and utilized.

There are a number of avenues which may be explored to reduce the costs of micropropagation such as mechanization/automation and photoautotrophic micropropagation (reviewed by Chu and Kurtz, 1990 and Kozai, 1991).

2.3 Red raspberry field productivity

In an established red raspberry plantation primocanes and floricanes compete for light, water, nutrients and assimilates (Vasilakakis and Dana, 1978; Williamson et al., 1979; Crandall, et al., 1980; Wright and Waister, 1982a, b; Braun and Garth, 1984; Braun, et al., 1989; Dale, 1989; Dalman, 1989). In a dense, natural stand, primocanes and floricanes inhibat the same space but may be temporally separated because of different growth rates (Whitney, 1982). In early spring, the carbohydrate reserves in the stems of overwintered primocanes are mainly used for the production of fruiting laterals and leaves on the laterals. The photosynthates produced by these early leaves are conserved in the stems initially and then translocated to the developing fruit later in the season. The carbohydrates reserved in the stems of floricanes become depleted by the end of the growing season resulting in senescence. The carbohydrates reserved in the root system are mainly used for primocane production. The primocanes channel carbohydrates back to the root system throughout the growing season (Whitney, 1982).

In cultivated plantations, inter-cane competition is artificially manipulated through various cane management systems. The appropriate cane management system depends on the cultivar, soil and climatic conditions. The best cane management system should maintain sufficient primocane growth and promote good yield and ease of harvest. The commonly used cane management systems include annual or biennial systems, hedgerow or stool systems and primocane vigor control (Shoemaker, 1978; Crandall and Daubeny, 1990).

The annual system allows primocanes to grow alongside the floricanes and competition between primocanes and floricanes exists throughout the season. The primocanes are then thinned and/or pruned to a desired density and height after growth cessation in the fall. Fruit are harvested every year. The biennial system allows either primocanes or floricanes to grow during a growing season. During the vegetative year only primocanes are allowed to grow and all primocanes are retained for fruiting in the following year. During the fruiting year only floricanes are allowed to grow and all primocanes are removed during the growing season. The competition between primocanes and floricanes is eliminated. Fruit are harvested every second year.

Nehrbas and Pritts (1988) found that fruit production of 'Titan', an average-vigor cultivar, was better using the annual system than the biennial system due to a reduction in fruiting laterals in the biennial system. However, Wright and

Waister (1982a, b) found that fruit production of 'Glen Clova' a relatively vigorous cultivar was better using the biennial system (7.74 t•ha^{-1}) compared with the annual system (3.8 t•ha^{-1}). The increased fruit yield with the biennial system was due to more canes which were taller with more fruit per cane. The biennial system increased leaf area and persistence and promoted a better pattern of light distribution, which reduced primocane shading, compared with the annual system. Apparently, cultivars with different degrees of vigor respond differently to the various management systems.

In the hedgerow system, primocanes are allowed to grow between hills to form a continuous row. The rows are narrowed down to a desired width (about 30 cm) by removing primocanes that develop outside the row. In the stool system, primocanes are allowed to grow around the parent canes to a distance of about 30 cm and those outside this radius are removed. The rows consist of discrete hills of red raspberry (Craig and Aalders, 1966; Mason, 1981; Agriculture Canada, 1984). Mason (1981) reported that 'Glen Clova' raspberry grown under the hedgerow system outyielded those under the stool system in 1972, 1973 and 1975 due to more canes in the hedgerow system. However, the stool system outyielded the hedgerow system in 1971 due to increased fruit per cane and greater mean berry weight (Mason, 1981). In Nova Scotia, the cultivar Trent yielded 66% more fruit when grown under the stool than the hedgerow system, while the cultivars Early Red, Malling

Promise, Carnival, Willamette and Viking did not respond to the management systems tested (Craig and Aalders, 1966).

Primocane vigor control is intended to control the growth of the first spring flush of primocanes by removing them when they reach a height of 10 - 15 cm. This minimizes the competition between the primocanes and floricanes in favor of floricane development, early in the growing season, while allowing the second flush of primocanes to grow. These primocanes are sufficient for good cropping in the following year (Lawson, 1980). The method of primocane suppression is either mechanical or chemical, including cutting, mowing and cultivation, or spraying desiccant such as Glufosinate on the young primocanes. Increased yields resulting from primocane vigor control were obtained using chemicals (Crandall et al., 1980) or cutting (Nehrbas and Pritts, 1988). Vigorous cultivars were more suited to vigor control than less vigorous cultivars. Similar results were reported by Williamson et al. (1979), Lawson (1980), Norton (1980), Buszard (1986), Freeman and Daubeny (1986), and Freeman et al. (1989). Red raspberry productivity is also affected by soil fertility, irrigation, soil management as well as genetic factors (reviewed by Mackerron, 1982; Dale, 1989; Crandall and Daubeny, 1990).

General recommendations for commercial red raspberry production in Eastern Canada were discussed by Agriculture Canada (1984). Briefly, the sites for establishing red raspberry fields should not have been used to grow tomato,

pepper, eggplant or potato in any of the previous 4 to 5 years. The ideal soil is deep, well drained sandy loam with high organic matter and a pH level of 5.7 to 6.0. Nitrogen at 70 $kg \cdot ha^{-1}$ may be supplied as 45% urea at 150 $kg \cdot ha^{-1}$ or ammonium nitrate at 200 $kg \cdot ha^{-1}$ in addition to barnyard manure at 35 to 45 $t \cdot ha^{-1}$ or poultry manure at 10 to 20 $t \cdot ha^{-1}$ before planting. Certified stock may be planted early in the spring or in the fall at 3 m between rows and 60 cm within rows. Weeds may be controlled by chemicals, cultivation or mulch as recommended by the local agriculture service. Hedgerow is the most commonly used system in Eastern Canada and the primocanes are thinned to about 10 to 20 per meter. Support by spread trellis system is provided to prevent cane breakage and make harvesting easier. The local small fruit protection guide should be followed with respect to pest management. The red raspberry cultivars recommended for Eastern Canada include Boyne, Festival, Killarney, Madawaska, Carnival, Gatineau, Newburgh and Comet. For growing 'Festival' in Quebec, annual cropping with a fruiting cane density of 10 $canes \cdot meter^{-1}$ under a hedgerow system is superior to biennial cropping, alternate side of the row cropping and chemical primocane vigor control (Buszard, 1986).

2.4 The field performance of micropropagated rosaceous fruit plants

Micropropagated fruit plants can be used only if the plants produced are true-to-type and genetically stable. It is well documented that MP plants posses a cultured-induced phenotype (CIP) which reflects acclimatization to the in vitro microenvironment (this will be discussed in section 2.5.1). The CIP reflects epigenetic variation. The anatomical and physiological changes that occur during acclimatization will be discussed in section 2.5. Acclimatized "field-ready" MP plants are anatomically and physiologically similar to conventionally propagated plants. Some of the epigenetic variation seen in MP plants are transient while others may persist up to several years following field planting. Some variations may be permanent and inheritable. These include growth characteristics and patterns which may or may not be desirable depending on the circumstances. Long-term field testing is required to confirm the genetic identity and stability of MP plants. However, such information is not widely available for most species. The following is a discussion of some of the transient phenotypic changes found in MP rosaceous fruit plants.

2.4.1 General vigor and growth habit

MP plants of a wide range of species grew more vigorously than CP plants under field conditions. These include blackberry (Swartz et al., 1983), strawberry (Damiano, 1980; Damiano et al., 1983; Swartz et al., 1981; Marcotrigiano et al., 1984; Cameron and Hancock, 1986; Cameron et al., 1985, 1989; Theiler-Hedtrich and Wolfensberger, 1987), apple (scion self-rooted) (Webster et al., 1986; Rosati and Gaggioli, 1987; Zimmerman and Miller, 1991), pear (*Pyrus communis* L.) (Zimmerman, 1991) and plum (*Prunus* spp.) (Howard et al., 1989b). This is a desirable response for the commercial propagators and commercial fruit growers since it enhances propagation efficiency and improves establishment of new plantations. The reason for the enhanced vigor of some MP plants is not clear. It may be related to the growth regulators used in the medium, the healthier status of MP plants (removal of viruses and other pathogens) or "rejuvenation effects" (see section 2.4.2). MP plants were usually actively growing whereas their CP counterparts were often dormant at field planting, making it difficult to draw valid comparisons of early vigor between the MP and CP plants. On the other hand, MP plants of peach rootstocks and self-rooted sour cherry were less vigorous compared to their CP counterparts during the first but not subsequent years (Rosati and Gaggioli, 1987). Self-rooted 'Gala' and 'Triple Red

Delicious' apple plants were less vigorous than CP material budded onto M. 7a rootstock until the third growing season when they grew more vigorously than the budded plants (Zimmerman and Steffens, 1989). This undesirable response lasted for only a short period of time. It may perhaps be overcome by developing management systems appropriate to the specific MP cultivar. The increased or decreased vigor responses were of short duration; lasting from a few months to a few years.

The growth habit of MP plants was usually more uniform than that of CP plants. For example, MP blackberry (Swartz et al., 1983) and strawberry (Swartz et al., 1981) had more uniform growth than their CP counterparts. Other changes in growth habit varied with the species but mostly related to increased vigor. MP blackberry had greater cane numbers, more lateral branching and more flowers compared with their CP counterparts (Swartz et al., 1983). MP strawberry plants produced more runners than runner propagated plants; the average increases were 50% depending on the cultivar (Swartz et al., 1981; Marcotrigiano et al., 1984; Bedard and Garneau, 1985; Cameron et al., 1985; Scott et al., 1985; Swartz and Lindstrom, 1986; Theiler-Hedtrich and Wolfensberger, 1987). Strawberry lateral bud activity was also enhanced by micropropagation resulting in a greater number of crowns and more flower trusses (Swartz and Lindstrom, 1986). Plants of MP strawberry were more compact

with multiple but smaller crowns and less and smaller leaves resulting in less yield per crown but more total yield per plant compared with CP plants (Swartz and Lindstrom, 1986; Cameron et al., 1989). Such changes in growth habit reflect more dry matter allocated to the reproductive structures such as crown, flower truss and fruit (Cameron et al., 1989). The response was temporary, lasting up to 1 year only, and was observed mainly on the mother plants and their first runners. The response was initially thought to be related to growth regulators used in the medium in vitro (Swartz et al., 1981). However, it might not relate to the in vitro cytokinin levels (Swartz and Lindstrom, 1986).

Hardwood cuttings taken from MP plants somehow rooted more easily for pear (Jones and Webster, 1989) and plum (Howard et al., 1989a) probably due to the "rejuvenation effects" of micropropagation (see section 2.4.3). Traditionally, hardwood cuttings from these species were difficult to root, micropropagation introduces new opportunities for enhancing nursery propagation efficiency for these species. Increased rooting of cuttings taken from MP plants was also observed in grape (Swartz and Lindstrom, 1986).

Increased vigor is a desirable feature for both nursery propagators and commercial fruit growers; it enhances the nursery propagation efficiency and improves the establishment of new plantations. Growth uniformity within the clones may

be due to the uniformity of their physiological status.

2.4.2 Rejuvenation

The behavior of MP plants under field conditions varied depending on the species. However, all MP plants exhibited some juvenile characteristics. This phenomenon was observed on MP blackberry (Zimmerman, 1986), gooseberry (Wainwright and Flegmann, 1984), strawberry (Swartz et al., 1981), apple (Zimmerman, 1986), peach (Rosati and Gaggioli, 1987) and grape (Zimmerman, 1986; Cancellier and Cossio, 1988) plants. The strawberry juvenile characteristics included increased runnering and branching, longer petioles and reduced photosynthetic activity (Swartz et al., 1981; Scott et al., 1985; Mohamed et al., 1991). This phenotype could be partly reversed by abscisic acid (ABA) application (Mohamed et al., 1991). However, MP plants were not truly juvenile because they could be induced to flower in vitro (Scorza, 1982). Although embryos derived from adult callus or tissue were always juvenile, MP plants initiated from adult tissue should be considered adult (Swartz, 1991). In fact, micropropagation could not rejuvenate all species as was found with avocado (Pliego-Alfaro and Murashige, 1987). Phytohormones were thought to be involved in this phenomenon probably due to their ability to alter characteristics related to juvenility (reviewed by Swartz, 1991). However, rejuvenation effects by

micropropagation have also been observed on plants cultured on growth regulator-free medium (Mohamed and Swartz, 1989). The reason for the rejuvenation effects of micropropagation are not completely clear.

Sometimes enhanced or delayed bloom dates occurred in MP strawberry (Swartz and Lindstrom, 1986).. Delayed blossoming was also reported in MP grape (Barlass and Skene, 1980), strawberry (Mohamed and Swartz, 1989), apple (Webster et al., 1985, 1986; Rosati and Gaggioli, 1987; Zimmerman and Stefens, 1989; Zimmerman and Miller, 1991) and cherry (Rosati and Gaggioli, 1987) plants. This may be the direct result of rejuvenation effects. Delayed blossoming may or may not be desirable depending on the circumstance. This response will be welcomed by strawberry propagators since it reduces the manual requirement to remove flowers in strawberry nurseries. Delayed blossoms may not be desired by the commercial fruit growers. However, this was not a concern for fruit growers since the commercial stock used was usually several generations from micropropagation.

2.4.3 Fruit production

The acceptability of MP plants by the commercial growers depends heavily on their productivity under field conditions. Field evaluation of a wide range of fruit species indicated that MP plants generally produced higher yields compared with

CP plants. MP blackberry yielded the same as standard plants the first fruiting season and some cultivars (Dirksen Thornless, Smoothstem and SI-US 68-6-17) yielded more the second fruiting season (Swartz et al., 1983). The increased fruit yield in MP blackberry was attributed to increased vigor and uniformity of growth. Generally, fruit sizes of MP plants were smaller than that of CP plants except for MP 'Dirksen Thornless' which had larger fruit (Swartz et al., 1983).

Increased fruit yield was also observed in MP strawberry (Damiano, 1980; Swartz et al., 1981; Cameron and Hancock, 1986; Cameron et al., 1985, 1989; Moore et al., 1991) and peach (Rosati and Gaggioli, 1987; Hammerschlag and Scorza, 1991). The increased fruit yield in MP strawberry was due to increased plant densities (Cameron et al., 1985, 1989). MP strawberry either had smaller (Swartz et al., 1981; Theiler-Hedtrich and Wolfensberger, 1987), same size (Cameron et al., 1989) or larger (Moore et al., 1991) fruit depending on the cultivars. MP 'Olympus' strawberry had larger fruit and the same yield the first year but the same fruit size and lower yield the second year as compared with runner plants (Moore et al., 1991). Significant variations in yield existed among MP subclones. However, they disappeared after 4 generations of runner propagation indicating a transient response. Increased yield on MP 'GF677' peach lasted for only 1 year (Rosati and Gaggioli, 1987). In some cases, early yield may be reduced as found on apple (Zimmerman and Steffens, 1989) and grape

(Cancellier and Cossio, 1988).

The increased vegetative vigor and uniformity of growth of MP plants did not always translate into greater fruit yields. However, MP plants performed similarly under field conditions with respect to fruit production; they produced more fruit or at least the same amount compared with CP plants. Where MP plants produced less and smaller fruit compared with CP plants this response was short in duration.

2.4.4 Sensitivity to agrichemicals

Agrichemicals such as herbicides, fungicides and insecticides as well as various plant growth regulators are used commonly in modern agricultural practices. MP plants grew differently under field conditions and responded to these chemicals differently compared with CP plants. Generally, MP plants, probably due to their juvenile growth characteristics, were more sensitive to some agrichemicals. MP red raspberry sometimes was more sensitive to certain herbicides (Meador, 1985; Neal et al., 1990). Neal et al. (1990) found that plants of MP 'Heritage' red raspberry were less tolerant to the herbicides Simazine and Orysalin during the first 4 weeks after planting but not after this. MP strawberry plants were also more sensitive to some agrichemicals, for example, the fungicide Plictran and the insecticide Buprimate, which caused leaf necrosis during acclimatization (Swartz and Lindstrom,

1986). Experiments are being carried out to establish appropriate management practices for MP red raspberry plants (Pritts, pers. com.).

2.4.5 Photosynthetic activities

Cultured plantlets have very low photosynthetic performance (see section 2.5.1). Photosynthetic performance of MP field plants has not been evaluated for most fruit species. Field planted MP strawberry 'Redchief' had higher net photosynthetic rates compared with CP plants (Cameron et al., 1989). However, in the same study, 'Earliglow' did not respond to micropropagation in the same way as 'Redchief'. This genotype-specific response lasted no more than three generations after micropropagation (Hancock, 1991).

2.4.6 Disease susceptibility

MP plants may be more susceptible to some diseases as a consequence of juvenile growth characteristics induced by micropropagation. However, most descriptions of field evaluations of MP plants lack this information. MP plants of susceptible strawberry cultivars were even more susceptible to red stele (*Phytophthora fragariae*) and verticillium wilt (*Verticillium albo-atrum*) while MP strawberry plants that experienced many subcultures in vitro were more susceptible to

mildew (*Sphaerotheca macularis*) (Shoemaker et al., 1985).

2.4.7 Genetic stability

The genetic stability of MP plants is very important. As discussed previously, most MP plant. variants involved transient phenotypic variations that were not detrimental mostly and lasted only a few months to several years. However, some cases of inheritable variation (somaclonal variants) did occur especially when adventitious regeneration was involved (Skirvin, 1976, 1978; Evans and Bravo, 1986; Swartz, 1991). It has been proposed that the risk of genetic variation may be minimized by using pre-organized explants (i.e. a meristem) and limiting the number of subcultures (Evans and Bravo, 1986). Despite the occurrence of off-type plants among MP plant populations, the rate of off-type plants from organized meristem tip-derived MP plants was less than or equal to that from conventional propagation (Swartz, 1991).

There has been limited research on the genetic stability of MP red raspberry. However, the incidence of variation was low and most variants were easily recognized and rogued. When 4,000 MP red raspberry plants were examined in the field over a 3 year interval, 24 plants were found with small deformed fruit (Feucht et al., 1985 cited in Snir, 1988). Only one chlorotic sectorial chimeral variant was observed in an MP blackberry population of about 1,800 plants examined during

the rooting stage (Swartz, et al., 1983). The typical MP strawberry variant, exhibited a narrow, sectorial, white-streaked leaf chlorosis, occurred at a very low rate (<0.1%) and was observed on only one of the eight meristem-tip lines (Swartz, et al., 1981). Rosati et al. (1986) considered MP loganberry plants to be true-to-type and genetically stable after examining 3,698 MP plants in the field for 3 years. Lewandowski (1991) claimed that after 3 years of intensive grape (*Vitis* spp.) production no changes in disease susceptibility or gross morphology were observed among MP populations. MP peach trees were phenotypically identical to the parent plants (Rosati and Gaggioli, 1987; Liverani et al., 1989).

2.4.8 Summary

Generally, most of the micropropagation-related epigenetic changes (increased vigor, runnering, suckering, branching, ease of rooting and delayed or reduced flowering) enhanced the plant propagation efficiency. Micropropagation was demonstrably very valuable to the nursery industry. On the other hand, the direct benefit of micropropagation to fruit growers was less clear. In fact, some of the micropropagation-related epigenetic changes (smaller fruit, susceptibility to disease and agrichemicals and delayed flowering) were undesirable to fruit growers.

Micropropagation has become an increasingly important technique for the agri-food industry. MP plants will become even more popular as we understand more about their behavior. As discussed previously, MP plants behaved differently in the field compared with CP plants. MP plants may respond differently to conventional management practices which may have to be modified or new ones developed to effectively utilize and maximize the benefit of micropropagation. This includes planting technique, weed control, disease and insect control, fertilization, irrigation and other cultural practice appropriate to the MP plants of specific species or cultivars.

2.5 Acclimatization of micropropagated plants

Micropropagated plantlets possess a culture induced phenotype (CIP) which includes plant miniaturization, decreased leaf epicuticular and cuticular waxes, reduced packing density of leaf mesophyll, increased percentage water content, reduced mechanical support tissues, non-functional stomata and low autotrophic metabolism. This phenotype prevents MP plantlets transferred directly from culture to ambient field or greenhouse conditions from growing normally without special handling. MP plantlets must undergo a period of acclimatization during which they are usually exposed to high relative humidity (RH) and relatively low light intensity for several days or weeks followed by a gradual adjustment of

RH and light to ambient levels. This process can be expensive in terms of labor and controlled environment facilities. Manipulation of the culture environment to alter the CIP towards photoautotrophy and hardening against water stress could abbreviate or eliminate the ex vitro acclimatization period and reduce the overall costs of micropropagation (reviewed by Kozai, 1991; Preece and Sutter, 1991; Donnelly and Tisdall, 1992).

2.5.1 The culture induced phenotype

The culture induced phenotype (CIP) is commonly observed on cultured plants and reflects acclimatization to the unique in vitro microenvironment. The CIP has been observed on a wide range of temperate species including apple (Brainerd and Fuchigami, 1981; Sutter, 1988), blackberry (Donnelly et al., 1986; Donnelly et al., 1987), blueberry (*Vaccinium* spp.) (Grout et al., 1986), cabbage (*Brassica oleracea* var. capitata) (Sutter and Langhans, 1982), carnation (*Dianthus caryophyllus* L.) (Sutter and Langhans, 1979), cauliflower (*Brassica oleracea* L. var. botrytis) (Grout and Crisp, 1977; Grout and Aston, 1978; Grout and Donkin, 1987), cherry (*Prunus avium x P. pseudocerasus* L.) (Sutter, 1985), chrysanthemum (*Chrysanthemum moriffolium* L.) (Wardle et al., 1983), plum (Brainerd et al., 1981), red raspberry (Donnelly and Vidaver, 1984a, b; Donnelly et al., 1984, 1985),

strawberry (Grout and Milliam, 1985; Fabbri et al., 1986; Donnelly et al., 1987) and sweetgum (*Liquidambar styraciflua* L.) (Wetzstein and Sommer, 1981; Wetzstein et al., 1981).

Typically, the in vitro microenvironment includes a saturated atmosphere (ca. 100% RH); extremely low CO_2 levels during the light period; and excessively high CO_2 levels during the dark period, due to limited gas exchange between the culture vessels and the outside atmosphere; exogenous carbohydrates and plant growth regulators in the media; relatively low light levels (ranging from 10 to 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and relatively high and constant temperature (20 - 28C; (reviewed by Zimmerman, 1988; Debergh, 1991; Kozai, 1991; Donnelly and Tisdall, 1992).

One of the typical features of the CIP was the miniaturization of cultured plants; leaves of cultured blackberry were only 1-2% the area of greenhouse-grown control plant leaves (Donnelly, et al., 1986). Similar observations were made on cultured red raspberry (Donnelly and Vidaver, 1984a) and Asian white birch (*Betula platyphylla* Rehd.) (Smith et al., 1986). Miniaturization may relate to cytokinins in the media. Larger organ size occurred during the rooting stage when cytokinins were excluded or substantially reduced. The smaller leaves may reduce water stress experienced by new ex vitro transplants. However, smaller leaf area would contribute to low total net photosynthetic rates too.

Plants grown under ambient conditions are commonly

covered with epicuticular waxes which act as a barrier to limit water loss by cuticular evapotranspiration. The effectiveness of this barrier depends on both the structure and amount (thickness) of deposited waxes. Cultured plants commonly have significantly less epicuticular wax than greenhouse- or field-grown plants resulting in the cultured plants being more vulnerable to desiccation. This phenomenon has been observed on a wide range of species including apple (Brainerd and Fuchigami, 1981), cabbage (Sutter and Langhan, 1982), carnation (Sutter, 1978; Sutter and Langhans, 1979; Ziv et al., 1987), cauliflower (Grout and Aston, 1977; Wardle et al., 1979; Short et al., 1987), chrysanthemum (Sutter, 1982), plum (Fuchigami et al., 1981) and strawberry (Fabbri et al., 1986).

In addition, epicuticular waxes on cultured leaves were structurally different from those on glaucous control leaves, lacking the characteristic crystalline structure of plates and rods. It has been suggested that the lack of epicuticular wax on cultured leaves was related to the high RH in culture vessels (Grout 1975; Grout and Aston, 1977; Wardle et al., 1983; Ziv, 1986) (see section 2.5.3). Low light intensity altered wax structure to contain more polar components which lead to more water loss in cultured cabbage (Sutter, 1984). The reduced and altered structure of foliar epicuticular waxes in cultured plants could result in excessive water loss through epicuticular transpiration.

Leaves of cultured plants are thinner with reduced or poorly defined palisade layers and loosely organized spongy mesophyll cells compared with leaves of control plants. Leaves of MP plum (Brainerd et al., 1981), red raspberry (Donnelly and Vidaver, 1984a), Asian white birch (Smith et al., 1986) and strawberry (Fabbri et al., 1986) had only one layer of palisade cells instead of two to three layers observed on control plant leaves grown in greenhouses or under field conditions. No clearly-defined palisade layer was present in leaves of MP cauliflower (Grout and Aston, 1978b) and sweetgum (Wetzstein and Sommer, 1982). These features, shared with "shade leaves" may result from development under relatively low light intensities in vitro.

Cultured plant tissues have increased percentage water content which may result from development under saturated atmospheres and low light intensities. Reduced mechanical support tissues in cultured red raspberry plants may be attributed to the tranquil in vitro conditions (Donnelly, et al., 1985). These features prevent MP plantlets from growing normally under ambient ex vitro conditions.

Several studies have revealed inferior vascular connections in petioles of Asian white birch (Smith et al., 1986), stems of carnation (Leshem, 1983) or the root-shoot interface of adventitious cauliflower plantlets (Grout and Aston, 1977). Poor vascular connection may intensify the water stress of new ex vitro transplants.

Stomata play an important role with respect to plant water relations because stomatal transpiration accounts for more than 50% of the total evapotranspiration during the day (Nobel, 1991). Stomatal transpiration rates were determined by stomatal index and apertures. Stomatal index was less sensitive to in vitro environment and remained consistent over a variety of conditions (Conner and Conner, 1984; Capellades et al., 1990). Stomatal index was slightly less on leaves of cultured plants than on leaves of control plants of *Leucaena leucocephala* (Lam) De Wit (Dhawan and Bhojwani, 1987). The size and shape of stomata on leaves of cultured plants differed from those on leaves of control plants. Stomata on leaves of cultured plants were usually larger as found on leaves of cultured apple (Brainerd and Fuchigami, 1981; Blanke and Belcher, 1989), chrysanthemum (Sutter, 1985), *Solanum laciniatum* L. (Conner and Conner, 1984), sour cherry (Marin and Gella, 1988; Marin et al., 1988) and sweetgum (Wetzstein and Sommer, 1983) and usually circular in shape as found on leaves of cultured apple (Brainerd and Fuchigami, 1981; Blanke and Belcher, 1989), carnation (Ziv et al., 1987), chrysanthemum (Sutter, 1985), sour cherry (Marin and Gella, 1988; Marin et al., 1988) and sweetgum (Wetzstein and Sommer, 1983) compared with those on leaves of control plants.

Most stomata on leaves of cultured cauliflower were probably not functional since they did not close in response to the stimulus (ABA at 10^{-4} M) (Wardle et al., 1979).

Stomata on leaves of cultured carnation did not close in response to stimuli such as darkness, ABA (10^{-4} M) or high osmolarity solutions (5 mM $CaCl_2$ or 0.8 M sucrose) (Ziv et al., 1987). Less than 5% of the stomata on leaves of cultured apple closed in response to 4 hours of darkness, ABA (10^{-7} M to 10^{-4} M), high osmolarity solution (1 M mannitol) or elevated CO_2 levels (0.12%) (Brainerd and Fuchigami, 1982).

The impaired function of stomata on leaves of cultured plants might result from mechanical failure. For example, the guard cell protoplasms of cultured carnation reacted normally to stimuli such as solution of various osmolarities or ABA although the stomata remained open (Ziv et al., 1987). Some stomata on leaves of cultured chrysanthemum (Wardle and Short, 1983), tart cherry (Marin et al., 1988), apple, cherry and sweetgum (Sutter, 1988) closed under low RH (45 - 65%) perhaps due to collapse of the guard cells. It was not tested whether or not they could re-open. The usually wide-apertured and non-functional stomata on leaves of cultured plantlets probably contributes significantly to the poorly regulated stomatal transpiration of ex vitro transplants.

Cultured shoots and plantlets are mixotrophic; they rely on exogenous carbohydrates from the medium as well as photosynthates (reviewed by Kozai, 1991). Cultured plantlets rely mainly on exogenous sugars (Conner and Thomas, 1981) as their photosynthetic competence is low; the maximum CO_2 uptake rates ranged from 0.86 $mg \cdot dm^{-2} \cdot hr^{-1}$ (or 31% that of

seedlings) in leaves of cultured cauliflower (Grout and Donkin, 1987) to 2.57 $mg \cdot dm^{-2} \cdot hr^{-1}$ (or 24.4% that of greenhouse control leaves) in leaves of cultured red raspberry (Donnelly and Vidaver, 1984b). Using ^{14}C labelling methods, Grout and Millam (1985) found that the photosynthetic capacities of leaves of cultured strawberry were about 10% that of new leaves formed 28 days ex vitro. Donnelly et al. (1984) found that the photosynthetic capacities of leaves of cultured red raspberry were 53 - 66% that of mature greenhouse control leaves. Cultured Asian white birch had photosynthetic capacities of about one third that of greenhouse-grown plants (Smith et al., 1986). The low autotrophic metabolism of cultured shoots and plantlets relates to the low CO_2 levels during the light period, the relatively low light intensities and the presence of exogenous carbohydrates in the media (reviewed by Kozai, 1991). The levels of photosynthetic electron transport in leaves of cultured cauliflower were comparable to those of seedlings. However, the total chlorophyll and Ribulose-1,5-bisphosphate carboxylase /oxygenase (Rubisco) activity were only 60% or 36%, respectively, of seedling leaves, indicating that some aspects of photosynthetic physiology were altered (Grout and Donkin, 1987).

Cultured plants with relatively high photosynthetic ability are considered photosynthetically competent species including Asian white birch (Smith et al., 1986), asparagus

(*Asparagus officinalis* L.) (Yue et al., 1992), chrysanthemum (Grout, 1988), potato (Grout, 1988) and red raspberry (Donnelly and Vidaver, 1984b; Donnelly et al., 1984). Cultured plants with relatively low or negative photosynthetic ability are considered photosynthetically incompetent species including cauliflower (Grout and Aston, 1978a) and strawberry (Grout and Millam, 1985). This low autotrophic metabolism may hinder the rapid adaptation of transplants to ex vitro conditions.

2.5.2 Ex vitro acclimatization

Decreased foliar wax and non-functional stomata with wide apertures lead to excessive water loss through evapotranspiration when cultured plantlets are transferred to the greenhouse or to field environments. Transplants may die of desiccation if proper steps, such as keeping the RH high, are not taken. Low photosynthetic competence or negative carbon balance of cultured plantlets impairs ex vitro growth and in more severe cases may lead to mortality (Grout and Donkin, 1987). Ex vitro acclimatization is a critical interval during which the MP transplants gradually acclimatize to ex vitro greenhouse or field environments under conditions of high RH and relatively low light intensity for 1 to several weeks. Control type anatomy and physiology develop gradually through a series of transitional organs during ex vitro

acclimatization. The first new leaves formed ex vitro were transitional with intermediate anatomy and physiology between the cultured and the control leaf phenotypes. The anatomical and physiological changes in new leaves formed during acclimatization included: increased leaf size and potential development, development of collenchyma in leaves, petioles and stems, increased trichome number, better organization of palisade and spongy mesophyll cells, increased foliar wax deposition, development of functional stomata as well as increased photosynthetic competence. These changes were gradual and involved transitional stages. During the period of adaptation to ex vitro conditions, water loss from leaves was reduced due to increased foliar wax deposition and functional stomata and the growth was promoted due to increased photosynthetic competence (reviewed by Zimmerman, 1988; Preece and Sutter, 1991; Donnelly and Tisdall, 1992).

Usually, the cultured plantlets were washed of the sugar-containing medium before transfer to potting mixtures. In some cases fungicides were used as dips or sprays to control fungal infection. Diluted water-soluble fertilizer was often applied on the foliage or as a soil drench at intervals to promote the growth of transplants. Maintaining conditions of high RH (ca. 90% or higher) was essential for the survival of MP transplants after they were removed from culture, especially during the first few days. High RH conditions were provided using mist or fogging systems, or polyethylene tents.

RH was then decreased gradually to ambient levels over a period of several days to several weeks, depending on the plant species and the ex vitro environment, by decreasing the frequency of misting or fogging or increasing openings in the tents. Higher light intensity than that used in vitro should be provided to promote photoautotrophy. Donnelly et al. (1985) found that the optimal survival rate for MP red raspberry was under light intensity three times higher than the in vitro level. The light intensity was then increased gradually over a period of several days to several weeks to ambient levels by providing more light or gradually removing the shade.

Persistent leaves were the critical organs for the survival of MP plants during acclimatization. They were the sites of transpiration and photosynthesis of transplants. The ex vitro behavior of persistent leaves depended on the plant species and the ex vitro environment. Generally, the persistent leaves changed little during their life span of a week to several months ex vitro before senescence. The persistent leaves of strawberry (Fabbri et al., 1986) and cauliflower (Grout and Aston, 1978b) transplants increased their size slightly due to cell elongation and enlargement. The persistent leaves of apple (Shackle et al., 1990), carnation (Sutter and Langhans, 1979), cauliflower (Grout and Aston, 1977; Wardle et al., 1979), plum (Fuchigami et al., 1981), strawberry (Fabbri et al., 1986), sweetgum (Sutter,

1988; Wetzstein and Sommer, 1983) and tart cherry (Marin et al., 1988) transplants acquired additional epicuticular wax deposition during acclimatization. This would tend to alleviate the water stress of transplants.

It was not clear whether stomata on persistent leaves could develop the ability to function. Some stomata on leaves of cultured apple (Brainerd and Fuchigami, 1981, 1982; Shackel et al., 1990), plum (Brainerd et al., 1981), tart cherry (Marin et al., 1988) and sweetgum (Wetzstein and Sommer, 1983) closed in response to certain stimuli (darkness, ABA, $CaCl_2$, sucrose, mannitol or elevated CO_2 levels) ex vitro. However, it was not made clear whether they could re-open. Stomata on persistent leaves of 21-day-old 'Silvan' blackberry transplants closed in response to 1 M NaCl and reopened when the stimulus was removed. However, stomata on younger persistent leaves did not close in response to 1 M NaCl (Tisdall, 1989). This indicated that stomata on persistent leaves of blackberry acquired the ability to function by 21 days ex vitro.

The photosynthetic capacity of persistent leaves on red raspberry transplants was very low and net respiration could occurred under some environmental conditions (Donnelly and Vidaver, 1984b; Donnelly et al., 1984). The photosynthetic rate of persistent leaves of strawberry transplants had increased 110% by 2 weeks ex vitro. However, it was only 20% that of new leaves formed 4 weeks ex vitro (Grout and Millam,

1985). The photosynthetic rate of persistent leaves of cauliflower (Grout and Aston, 1978; Grout and Donkin, 1987) and Asian white birch (Smith et al., 1986) transplants did not increase considerably with time during acclimatization. The contribution of persistent leaves to the growth of transplants involved both their photosynthetic and storage function. However, the magnitude of each contribution was not clear; probably depending on the plant species and the ex vitro environment.

The functionality of in vitro-formed roots has not been fully evaluated. It was commonly observed that transplant growth was significantly enhanced when roots were present at the time of transplantation. This was true for both cultured blackberry (Tisdall, 1989) and potato (Leclerc, 1990).

New leaves formed ex vitro had increasingly larger size, higher photosynthetic capacity, greater ability to regulate water loss (due to more epicuticular wax deposition and increasingly functional stomata); they were transitional leaves. These leaves had anatomy and physiology intermediate between the in vitro-formed and the greenhouse-grown control plant leaves. Red raspberry leaves formed successively ex vitro had increasingly higher photosynthetic capacity and the fifth or sixth new leaves approached that of the control leaves (Donnelly and Vidaver, 1984b). Stomata with increasing ability to function were observed on transitional leaves of apple (Brainerd et al., 1981), cauliflower (Wardle et al.,

1979) and cherry transplants (Marin, et al., 1988). Until the stomatal closure mechanism was well developed, water loss from leaves could not be effectively regulated.

Ex vitro acclimatization of grape was enhanced by CO_2 enrichment from ambient levels to 900 - 1500 ppm (Lakso et al., 1986). Ex vitro acclimatization of asparagus (Desjardins, 1990) and strawberry (Desjardins et al., 1987) was enhanced by CO_2 enrichment from ambient levels to 900 - 1500 ppm with supplemental light up to 80 - 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. There was a synergistic relationship between CO_2 enrichment and supplementary lighting in promoting the growth and survival of MP transplants. The best results were obtained from treatments of CO_2 enrichment with supplementary lighting. These treatments reduced the need for misting by 15 to 21 days but successful transplantation was still dependent on high RH.

Acclimatization was fully achieved when transplants had several new leaves formed ex vitro with functional stomata to regulate transpiration, active photosynthesis to provide a positive carbon balance and strong root systems to provide uptake of sufficient water and mineral nutrients. Only fully acclimatized MP transplants can be established successfully under field conditions.

2.5.3 In vitro hardening-off

As discussed previously, micropropagated plantlets must undergo a period of ex vitro acclimatization due to the CIP. The CIP reflects acclimatization to the unique in vitro microenvironment. The typical in vitro microenvironment includes a saturated atmosphere (ca. 100% RH), limited gas exchange between the culture vessels and the ambient atmosphere, exogenous carbohydrates and relatively low light intensity. Modification of this environment towards ambient conditions is one possible way to promote control-type anatomy and physiology of cultured plantlets. If full or partial hardening can be done in vitro the ex vitro acclimatization period could be substantially shortened or completely eliminated thus reducing the overall costs of micropropagation.

2.5.3.1 Relative humidity

In micropropagation, culture vessels were usually closed to prevent desiccation and to avoid contamination. However, this practice results in saturated RH (100%) due to accumulation of moisture from evaporation of the medium and transpiration of cultured shoots. This high RH may be advantageous for the survival and growth of the delicate, fragile explants and shoots in culture but it is detrimental

for the normal development of foliar wax and stomata. As discussed previously, leaves of cultured plantlets generally had less foliar wax compared with leaves of control plants and this has been linked to conditions of high in vitro RH. Wax deposition on leaves of cultured chrysanthemum (Sutter, 1982) and carnation (Sutter and Langhans, 1979) plantlets was found to be minimal or nil at saturated in vitro RH (100% RH). Increased foliar wax deposition on cultured plantlets under various conditions of reduced in vitro RH were achieved in a wide range of species. Glaucous cabbage plantlets with structured waxes could be induced at considerably lower RH (35%), although, the plantlet growth was severely hindered (Sutter and Langhans, 1982). Greenhouse-grown carnation was covered with dense foliar wax rods whereas in vitro cultured carnation lacked structured foliar waxes (Sutter and Langhans, 1979). Some foliar waxes developed on cultured leaves of carnation after exposure to 70% RH or lower but not at 80% for 7 days (Ziv, 1986). A negative relationship between in vitro RH and the amount of foliar wax on leaves of cultured cauliflower was noted by Short et al. (1987). They found that the leaves of cultured cauliflower had less foliar wax when grown under saturated RH (20 $mg \cdot cm^{-2}$) compared with plantlets grown under 80% RH (49.4 $mg \cdot cm^{-2}$). Well developed foliar epicuticular waxes were observed on cauliflower plantlets cultured under reduced in vitro RH (30%) brought about by covering the media with lanolin (Wardle et al., 1983). Rose

(*Rosa multiflora* L. 'Montse') plantlets cultured under 75% RH and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity had foliar waxes similar to leaves of greenhouse-grown rose plants (Capellades et al., 1990).

Higher stomatal density was found on leaves of cucumber (*Cucumis sativus* L.), tomato and green pepper grown under high humidity compared to those grown under lower RH (Bakker, 1991). Stomata on leaves of cultured plants grown under saturated RH were usually larger, circular and not functional compared with the normal functional stomata on control leaves grown under much lower RH. Improved stomatal anatomy and functioning was obtained on a wide range of species by reducing in vitro RH. Stomata on leaves of chrysanthemum plantlets grown under reduced RH (33%) had narrower apertures than those grown under saturated RH (Wardle et al., 1983). Narrower stomatal apertures reduced transpiration losses and increased the survival rates at transplantation (Wardle et al., 1983). Rose plantlets cultured under 75% RH and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ had stomata similar to those on leaves of greenhouse-grown plants (Capellades et al., 1990). Stomata on leaves of grape plantlets cultured under 94% RH closed following exposure to 20% RH for 18 hours in darkness whereas those cultured under 100% RH remained open (Smith et al., 1992).

Daily brief exposure to low RH, by uncovering the plantlets for various time periods, increased the survival

rates of cultured cherry plantlets during ex vitro acclimatization compared with control plantlets not subjected to low RH. This suggested that brief exposure to low RH was somehow stimulating (Marin and Gella, 1987). Improved water relations of cultured grape plantlets under reduced RH (94%) was further improved by the addition of paclobutrazol to the medium at 0.5 - 1 $mg \cdot liter^{-1}$ (Smith et al., 1992).

Lowering the RH in culture vessels could be achieved by using desiccant in or around the culture vessels (Wardle et al., 1983; Ziv, et al., 1983; Short et al., 1987); using oily medium overlays such as vegetable shortening, lanolin, paraffin, or mineral oil (Sutter and Langhans, 1982; Ziv, et al., 1983; Short et al., 1987; Crane and Hughes, 1990); opening culture vessels to low RH atmospheres for various durations (Brainerd and Fuchigami, 1981); using special vessel closures that facilitated water loss (Fari et al., 1987; Short et al., 1987; Kozai, 1988a,b; Kozai et al., 1988b; Smith et al., 1990b); cooling the bottom of culture vessels (Vanderschaeghe and Debergh, 1988; Capellades et al., 1990), increasing the agar concentration (Ziv, 1986; Rhaman and Blake, 1988) or using special incubation chambers that provided controlled environments (Kozai et al., 1987; Fujiwara et al., 1988). However, precisely controlling in vitro RH for an extended period of time was still difficult. Medium desiccation must be considered if reduced in vitro RH was accomplished by forced ventilation (Walker et al., 1988).

While the anatomy and physiology of cultured plants were improved by reduced in vitro RH, growth and multiplication rates declined, especially when the RH was below 80-85% (Ziv, et al., 1983; Ziv, 1986; Short et al., 1987). The disadvantage associated with low in vitro RH for cultured chrysanthemum and carnation was the deleterious effect on plantlet growth, especially root growth (Wardle et al., 1983). Short et al. (1987) also found that the growth of cultured cauliflower and chrysanthemum plantlets were severely hindered when the RHs were reduced to 70% or lower. The optimal in vitro RH for improving foliar wax deposition and stomatal function while maintaining good growth and multiplication rates has yet to be determined and may vary from species to species.

2.5.3.2 Carbon dioxide

Culture vessel closures limit gas exchange between the culture vessels and the ambient atmosphere. This results in extremely low CO_2 levels in the culture vessels during the light period, probably due to depletion by photosynthesis, and excessively high CO_2 levels during the dark period probably due to accumulation from respiration (Desjardins et al., 1988; Infante et al., 1989; reviewed by Kozai, 1991; Preece and Sutter, 1991; Donnelly and Tisdall, 1992).

CO_2 levels in culture vessels were depleted to the

compensation point (≤100 ppm in most cases) within 2 to 3 hours after the onset of light and remained low during the entire light period for more than 8 genera of ornamental plantlets (Fujiwara et al., 1987; Infante et al., 1989). This indicated that the cultured shoots were actively photosynthesizing and the photosynthetic rates of plantlets in vitro were limited by low CO_2 levels in the culture vessels during the light period. Increasing the CO_2 levels in culture vessels, especially during the light period, seemed to be a logical step to promote the photosynthesis of cultured plants. Improved growth and increased photosynthesis by CO_2 enrichment in vitro has been accomplished for a wide range of species, including carnation (Kozai, 1988a,b; Kozai and Iwanami, 1988), orchid (Kozai et al., 1987), potato (Kozai et al., 1988a), strawberry (Desjardins, 1990; Kozai et al., 1991a), *Actinidia deliciosa* L. (Infante et al., 1989) and tobacco (*Nicotiana tabacum* L.) (Kozai et al., 1990c). Detached axillary shoots of cacao (*Theobroma cacao* L.) could only be induced to elongate and produce normal leaves and sometimes roots under 20,000 ppm CO_2 and 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light (Figueira et al., 1991).

The fresh weight of carnation plantlets grown under enriched CO_2 (1,000 to 1,500 ppm in the culture room) was doubled that of those grown under ambient CO_2 at 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ after 30 days of incubation (Kozai and Iwanami, 1988). The dry weight of orchid plantlets grown under enriched CO_2 (950

to 1,000 ppm in acrylic chambers) was twice that of plantlets grown under ambient CO_2 at 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$ after 70 days of incubation (Kozai et al., 1987). The net photosynthetic rates of potato shoots cultured under enriched CO_2 (CO_2 levels inside culture vessels were maintained at 350 - 500 ppm by adjusting the CO_2 levels in the culture room to between 500 to 3,000 ppm) in conjunction with higher light intensity (210 to 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were significantly higher compared to those under ambient CO_2 and light conditions (Kozai et al., 1988a),

The growth and net photosynthetic rates of strawberry plantlets cultured in the "photoautotrophic tissue cultured system" which provided 300 - 330 ppm CO_2 levels, 96 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 93% RH were 72% and 775% higher, respectively, compared with plantlets grown under standard tissue culture conditions which provided 150 - 240 ppm CO_2 levels, 42 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and saturated RH (Fujiwara et al., 1988). The fresh weight of strawberry plantlets grown on sugar-free medium under enriched CO_2 (2,000 ppm in the culture room during the light period) was 86% and 59% heavier, respectively, than those grown at ambient CO_2 on media with 0 or 20 $g \cdot liter^{-1}$ sucrose (Kozai et al., 1991a),

The photosynthetic rates of cultured *Actinidia deliciosa* L. were 4 times higher under 1,450 or 4,500 ppm CO_2 compared with those grown under ambient CO_2 (Infante et al., 1989). The dry weight of tobacco plantlets grown under enriched CO_2 (700 to 4,200 ppm in the culture room) was more than twice

that of plantlets grown under ambient CO_2 regardless of light intensity (from 61 to 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and with (20 $g \cdot liter^{-1}$) or without sucrose after 30 days of incubation (Kozai et al., 1990c).

The effect of CO_2 levels on the development and functional capacity of stomata was less clear. Stomatal conductance of apricot (*Prunus armeniaca* L.) decreased by 90% when the CO_2 concentration was increased from 340 ppm to 1,000 ppm indicating stomatal closure. However, the stomata reopened when the CO_2 levels were increased above 1,000 ppm (Robinson et al., 1988).

In vitro CO_2 enrichment could be achieved using a gas permeable film for vessel closures (light transparent polymethylpentene with a 0.02 μm porous polypropylene path, 8 mm in diameter, in the center) (Kozai et al., 1988b, 1990a, 1991a; Kozai, 1988a), increasing CO_2 levels in the culture room (Kozai et al., 1988a, 1990b, c, 1991a, b; Kozai and Iwanami, 1988), directly supplying CO_2 to culture vessels (Fujiwara et al., 1988; Kozai, 1988b; Kozai, et al., 1988b; Kozai, 1991) or by culturing plants in CO_2 enriched incubation chambers (Kozai et al., 1987; Fujiwara et al., 1988; Walker et al., 1988; Laforge et al., 1990, 1991). The CO_2 levels in loosely capped or film-covered culture vessels could be maintained at 300 - 350 ppm if the CO_2 levels in the culture room were kept above 500 ppm (Fujiwara, et al., 1987). Pure or diluted CO_2 gas could also be supplied directly into

culture vessels, although larger culture vessels (with about 1,000 ml or more air volume) were more effective for such a system (Fujiwara, et al., 1988). The optimal in vitro CO_2 levels for improving stomatal function and increasing photosynthetic capacity have yet to be determined.

Several apparati have been developed to provide control of environmental conditions for studying their effects on the growth of in vitro cultured plants (Kozai and Iwanami, 1988; Laforge et al., 1990). Kozai and Iwanami (1988) placed transparent acrylic boxes (83 x 50 x 34 cm, length x width x height) with attached gas supply systems into plant growth chambers. Carbon dioxide in each box was then enriched to the desired levels through a CO_2 control unit. The gas mixtures in the boxes were further mixed by a micro fan. No devices were installed to regulate the temperature or RH inside the boxes. Thus this apparatus can only be used to study the effects of CO_2 levels on the growth of in vitro cultured plants. Laforge et al. (1990) built miniature growth chambers (56 x 42 x 30 cm, length x width x height) within 65 liter polyethylene chambers with air-tight plexiglass covers. The chambers were equipped with a CO_2 gas mixture supply system, water-to-air heat exchanger and 2 fans. These miniature growth chambers may be used to study the effects of CO_2 levels, temperature and light intensity but not RH on the growth of in vitro cultured plants.

The common deficiency of these two apparatus is their

inability to control the RH levels inside the chambers (or boxes). In addition, the environment inside the boxes/chambers was not sterile and the culture vessels were capped as in standard micropropagation practice thus the environmental conditions inside the culture vessels could not be effectively controlled.

Smith et al. (1990b) rooted in vitro cultured chrysanthemum plants in cellulose plugs (Sorbarods) in vessels with circular holes sealed with Tyvek Europeel T. Tyvek Europeel T is spunbonded polyolfin that acts as a bacteriolgical barrier while allowing water vapour to diffuse through. Smith et al., (1990b) obtained RHs inside the vessels of 96 and 94% respectively, with 5 holes of either 14 or 20 mm diameter along the wall of the vessels. However, they did not control other environmental conditions such as CO_2 or temperature.

2.5.3.3 Carbohydrate source

Carbohydrates are essential elements in culture media providing the cultured plants with a carbon and energy source. Sucrose is the most commonly used carbohydrate and is usually added to medium at 2 to 3% (Conner and Thomas, 1981; George and Sherington, 1984; Langford and Wainright, 1987). Sucrose in the medium generally promoted the growth of cultured plants. For example, shoot and root growth of jackfruit

(*Artocarpus heterophyllus* Lam.) were increased linearly when the sucrose in the medium was increased from 0 to 20 $g \cdot liter^{-1}$ (Rahman and Blake, 1988). Rose propagules died if sugar was omitted from the medium (Langford and Wainwright, 1988). There was a positive relationship between the growth of in vitro rose plantlets and sugar concentration in the media up to 40 $g \cdot liter^{-1}$ (Langford and Wainwright, 1988). A similar response was found in potato shoots when cultured on media with sucrose ranging from 0 to 30 $g \cdot liter^{-1}$ (Kozai et al., 1988b). The growth of rose plantlets was hindered if sugar concentration was less than 10 $g \cdot liter^{-1}$ or more than 40 $g \cdot liter^{-1}$ (Langford and Wainwright, 1988). However, sucrose had adverse effects on the development of the photosynthetic apparatus and on the biosynthesis of chlorophyll (Van Huystee, 1977; Wetherell, 1982). There was a negative relationship between the CO_2-uptake-ability of plantlets and the medium sucrose concentration they were grown in. The CO_2 uptake rates of rose propagules (cv Iceberg) grown on media containing 10, 20 or 40 $g \cdot liter^{-1}$ sucrose were 20.77, 17.46 and 11.32 μmol CO_2 $h^{-1} \cdot g^{-1}$ fresh weight, respectively (Langford and Wainwright, 1988). The maximum net photosynthetic rates of rose plantlets cultured on a 1% sucrose medium were 10 times higher than on a 5% sucrose medium (Capellades et al., 1991). It has been suggested that inhibition of photosynthesis by sucrose in the medium might be related to the low rate of regeneration of the carboxylation substrate Ribulose-1,5-

bisphosphate (RuBP) due to the accumulation of soluble sugars in the leaves and the inhibition of Rubisco activity (Capellades et al., 1991; Grout and Donkin, 1987).

Wetmore and Rier (1963) found that tracheary element production in *Parthenocissus tricuspidata* L. callus varied quantitatively with changes in sucrose concentration provided that auxin was present in the media. It appears that sucrose was necessary for the development of the vascular system in adventitious cultures. Exogenous sucrose may not be the only source of available carbohydrate. Photosynthates produced by the leaves, although limited in quantity, also serve as a carbohydrate source. The implication is that the exogenous sucrose may be reduced or omitted provided that the cultured leaves have a photosynthetic rate high enough to sustain a positive carbon balance.

It was suggested that leaves of some plants, such as chrysanthemum and cauliflower, formed during culture acted mainly as storage organs providing nutrients to new leaves developed during acclimatization ex vitro (Wardle et al., 1979, 1983). This was confirmed by tracing the redistribution of rubidium (Rb) in cultured plantlets transferred to soil. Grout and Millam (1985) also found that in vitro formed strawberry leaves acted as storage organs and nutrient sources during acclimatization. Thus large plantlets survived and established better than small ones when they were transferred to ex vitro conditions.

Many species can be cultured on sugar-free medium. For example, carnation (Kozai and Iwanami, 1988; Kozai et al., 1987, 1988b), cauliflower (Grout and Donkin, 1987), Chinese mustard (*Brassica campestris* L.) (Kozai et al., 1991b), jackfruit (Rahman and Blake, 1988), orchid (Kozai et al., 1987), potato (Kozai et al., 1988a) and strawberry (Fujiwara et al., 1988b; Kozai et al., 1991a) plantlets could grow photoautotrophically. In fact, autotrophy was promoted in cultured plantlets of carnation (Kozai and Iwanami, 1988), cauliflower (Grout and Donkin, 1987; Short et al., 1987), potato (Kozai et al., 1988a) rose (Langford and Wainwright, 1987; Capellades et al., 1991) and strawberry (Kozai et al., 1991a) on sucrose-free or reduced sucrose medium. Sucrose in the medium had adverse effects on the photosynthesis of cauliflower (Grout and Donkin, 1987), rose (Langford and Wainwright, 1987; Capellades et al., 1991) and tobacco (Kozai, 1990), but promoted the growth of cultured plantlets (Honjo et al., 1988). Potato shoots could grow on sugar-free medium and their growth exceeded that of shoots on sucrose containing medium when the light intensity was increased to (210 or 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and CO_2 levels were maintained at 350 - 500 ppm by adjusting the CO_2 levels in the culture room to between 500 to 3,000 ppm (Kozai et al., 1988a).

Reducing the sucrose concentration of the medium to promote the photosynthetic capacity of plantlets may be limited to the rooting stage (Stage III) because maximum in

vitro plantlet growth is necessary during the multiplication stage (Stage II). Minimizing the exogenous supply of carbohydrate may benefit both the development of the photosynthetic apparatus and chlorophyll synthesis.

2.5.3.4 Light

The light intensity in culture vessels was generally relatively low, ranging from less than 10 to around 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This low light intensity contributes to the CIP. Generally, increasing the in vitro light intensity promoted the growth and photosynthesis of cultured plants, especially in conjunction with CO_2 enrichment. This has been observed in a wide range of species including Chinese mustard (Kozai et al., 1991b), carnation (Kozai and Iwanami, 1988; Kozai et al., 1990a), orchid (Kozai et al., 1990b), potato (Kozai et al., 1988a), strawberry (Fujiwara et al., 1988) and tobacco (Kozai et al., 1990c) when light intensity was increased from 60 up to 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The growth and photosynthetic rates of potato shoots were more than doubled under higher light intensity (210 and 400 compared with 65 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in conjunction with enriched CO_2 (Kozai et al., 1988a).

Increasing the light intensity alone might not be effective in increasing the photosynthesis of plantlets growing at ambient CO_2 conditions. For example, the net photosynthetic rates of orchid (Kozai et al., 1987) and

tobacco (Kozai, 1990) plantlets were similar when the light intensity was increased from 51 to 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under ambient CO_2 conditions. Cultured plantlets might have light saturation points well below that of seedling or control plants. The photosynthetic rates of cultured Asian white birch did not change when the light intensity was increased from 200 to 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Smith et al., 1986) indicating that their light saturation point was at or below 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The fresh weights of potato plantlets did not change when the light intensity was increased from 210 to 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 29 days (Kozai et al., 1988a). The growth of cultured *Actinidia deliciosa* L. was promoted under light intensity of up to 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ but detrimental effects occurred at higher light intensity (Infante et al., 1989). The photosynthetic rates of cultured plantlets can be further increased when both light and CO_2 are supplemented (Kozai, et al., 1987; Desjardins et al., 1988).

Decreasing oxygen levels in vitro to reduce the photorespiration rate can also help to increase photosynthetic productivity (Shimada et al., 1988). The net photosynthetic rates of *Primula malacoides* L. and chrysanthemum cultured under 1% or 10% O_2 were 3 or 1.5 times higher, respectively, than those grown under 21% O_2 (Shimada et al., 1988). The potential exists to increase the photosynthetic rates of cultured plants substantially provided that the physical environment is properly controlled (Kozai, 1991).

2.5.3.5 Summary

The CIP is due to the unique in vitro environment. The growth and photosynthetic capacity of cultured plants is mainly limited by high RH, low CO_2 levels, exogenous carbohydrate and low light intensity. . It was possible to promote control-type anatomy and physiology in cultured plants by modifying the in vitro environment toward ambient levels. Plantlets in vitro could be hardened by reducing the RH, increasing the CO_2 and light intensity and reducing or eliminating sucrose in the medium. Kozai (1991) reviewed the measurement and control of microenvironments in vitro. Short et al. (1987) obtained hardened cauliflower and chrysanthemum plantlets grown under 80% RH by using partly closed screw top lids. They found that these plantlets had photosynthetic capacities comparable to those of seedlings when sucrose was excluded from the medium.

Photoautotrophic micropropagation is possible through optimization of in vitro conditions to promote control-type anatomy and physiology. This directly reduces the cost of materials, such as sucrose, and reduces the biological contamination rate. While this has the potential to increase productivity and plantlet quality it changes the nature of micropropagation as we know it. In vitro hardened photoautotrophic plantlets would require fewer changes anatomically and physiologically to adapt to ex vitro ambient

conditions. Greater ex vitro growth and survival rates might therefore be expected. There are a number of other avenues which may be explored to facilitate the in vitro hardening of cultured plants. Cellulose plugs have been used to replace agar with resulting improvements in plantlet growth and ex vitro transplantation (Roberts and Smith, 1990). The addition of osmotic agents such as polyethylene glycol (PEG) (Short et al., 1987), plant growth regulators such as paclobutrazol (Smith et al., 1990a,b, 1991, 1992) or elevated levels of agar (Ziv, et al., 1983) also improved in vitro hardening.

Preface to Chapter 3

Chapter 3 is a reproduction of a manuscript by R. Deng, D.J. Donnelly, and D.J. Buszard which was presented orally by R. Deng at the 1992 Symposium of the International Society for Horticulture Science on In Vitro Culture and Horticultural Breeding. This was held in Baltimore, Maryland, U.S.A. from June 28 to July 2, 1992. This article is now in press in Acta Horticulturae. This chapter gives a comprehensive discussion of the field performance of micropropagated and conventionally propagated red raspberry in Quebec, Canada.

Chapter 3 Field performance of micropropagated and conventionally propagated red raspberry under hedgerow and stool systems.

3.1 Abstract

Field performance of micropropagated (MP) and conventionally propagated (CP) red raspberry (*Rubus idaeus* L. cv. Comet and Festival) was examined under hedgerow and stool cane management systems for 3 seasons (1989 to 1991) in Montreal, Canada. All MP plants survived the planting year, but only 58% of CP plants survived field transplantation and 92% of CP plants survived after replanting. MP plants established better and were more vigorous than CP plants during this study as indicated by greater cane height, increased number of leaves per cane and more and larger primocanes per plant in 1989 and more primocanes per plant in 1990 and 1991. MP plants required comparatively more thinning than CP plants in both 1990 and 1991. Leaf and stem morphology were consistent within each cultivar regardless of propagation methods and plant sources. MP 'Festival' in 1990 yielded 2.2 $MT \cdot ha^{-1}$, almost half the amount of established commercial plantings in Quebec, while yields from CP 'Festival' and MP and CP 'Comet' were negligible. The MP 'Festival' crop (8.42 $MT \cdot ha^{-1}$) also outyielded CP 'Festival' (6.8 $MT \cdot ha^{-1}$) and both MP (5.72 $MT \cdot ha^{-1}$) and CP (4.91 $MT \cdot ha^{-1}$)

'Comet' in the second fruiting year. The increased fruit yields in MP 'Festival' plants might be derived partly from greater numbers of flowers and fruit on longer laterals. However, yields of 'Comet' were unaffected by propagation method. Winter hardiness and photosynthetic capacity of primocanes and floricanes were unaffected by propagation method and cultivar and cane management systems. Cane management systems had no effect on field performance of MP or CP plants in this relatively young plantation. MP plants, produced in our laboratory or purchased from a commercial micropropagator, were superior to CP bare-rooted canes obtained from a certified grower for both nursery propagation and fruit production due to their better establishment and increased vigor.

3.2 Introduction

Red raspberry (*Rubus idaeus* L.) has biennial stems and perennial roots and is conventionally propagated (CP) primarily by root suckers (Whitney, 1982; Jennings, 1988; Crandall and Daubeny, 1990). Root cuttings (Torre and Barritt, 1979) and leafy summer cuttings (Howard et al., 1987) can also be used to establish new plants. Certified planting stock is usually produced through CP methods by designated nursery propagators who strictly follow certification program guidelines (Buonassisi et al., 1989; Dale and Vandenberg,

1989; Ellis et al., 1991). In some cases, CP methods can not meet the demand for planting stock of the difficult to propagate, or newly released cultivars (Cormack et al., 1976; Lawson and Wiseman, 1980; Snir, 1981; Swartz and Lindstrom, 1986).

Micropropagation of healthy red raspberry can produce plants far more quickly than conventional means and the operation can be carried out year-round (Donnelly and Daubeny, 1986; Snir, 1988). Despite the advantages, micropropagation is relatively more expensive than conventional methods (Zimmerman, 1986) and comparative long-term field performance data are lacking (Dale, 1989). In addition, MP plants may respond to cultural practices differently from CP plants (Trinka and Pritts, 1990). Comparative field evaluations of CP and MP red raspberry are prerequisite to recommendations regarding the use of micropropagation for nursery propagation and commercial fruit production. Field examination of MP blackberry (*Rubus occidentralis* L.) (Swartz et al., 1983) and strawberry (*Fragaria x ananassa* Duch.) (Swartz et al., 1981; Damiano et al., 1983; Grout and Millam, 1985; Cameron et al., 1985; Theiler-Hedtrich and Wolfensberger, 1987) indicated that MP plants performed as well or better than and were phenotypically similar to their CP counterparts.

The objectives of this study were to examine the field establishment, cane growth and development, morphology, winter kill and die-back, photosynthesis and fruit yield of MP and CP

'Comet' and 'Festival' red raspberry under hedgerow and stool cane management systems in Montreal, Canada.

3.3 Materials and methods

MP raspberry were from two sources. .'Comet' was produced in our laboratory as previously described (Donnelly et al., 1980). Rooted plantlets were acclimatized for 4 weeks in the greenhouse and hardened-off in an outdoor cold frame prior to field planting. MP 'Comet' canes averaged 21 cm in height with 11 leaves when transferred to the field. MP 'Festival' were purchased from Nourse Farms (S. Deerfield, MA) as "acclimatized and field-ready". MP 'Festival' canes averaged 3 cm in height with 4 leaves when transferred to the field. Certified CP plants of both cultivars were purchased from Pepiniere A. Masse Inc., Quebec, a certified propagator. Dormant CP canes averaged 25 cm in height with white buds 1 - 3 cm long present on the bases at the time of field planting.

MP and CP 'Comet' and 'Festival' red raspberry were observed in the field from 5 June 1989 to 1 October 1991. Planting and cultural practices followed the recommendations of Agriculture Canada (1984). Weed control was achieved using mechanical means only. Overhead irrigation was applied as needed. The soil was a silt loam and the previous crop was pumpkin (*Cucurbita pepo* L.). The spacing between rows was 2.75 m and within rows was 0.5 m. Each plot was a single row

3 m long with 6 plants. One guard row was planted around the experimental field. The 2 cultivars (Comet and Festival), 2 propagation methods (MP and CP) and 2 cane management systems (stool and hedgerow) were arranged in a 2 x 2 x 2 factorial experiment according to a randomized complete block design (RCBD) with 6 blocks.

Survival rates were recorded 45 and 100 days after planting and in May 1990. Growth parameters including cane heights, leaf number per cane, cane number per plant, cane diameters (10 cm above ground level), net photosynthetic rates of primocane and floricane leaves, total fruit yield and fruit weight (mean of 25 fruit) as well as the overall appearance of primocanes and floricanes were recorded from early summer throughout the growing seasons of 1989, 1990 and 1991. The absolute minimum (January) and maximum (July) temperatures in 1989, 1990 and 1991 were -27, -23.7 and -26.5C; and 33.4, 31.0, and 33.3C, respectively.

Cane heights and leaf numbers per cane were recorded on four canes, randomly chosen from the four central plants in each plot, and cane number per plant were recorded on four central plants per plot at biweekly intervals from early summer throughout the growing seasons of 1989, 1990 and 1991. Cane diameters were measured with a caliper at 10 cm above ground level in late fall on 4 (1989) or 10 (1990, 1991) canes randomly sampled from 4 central plants per plot. In 1990 and 1991 primocanes were thinned to about 30 canes per plot and

the floricanes removed by 2 weeks after harvesting. Total primocane and floricane numbers and their mean fresh and dry weights were determined.

Net CO_2 assimilation rates of primocane leaves were measured on the two most recently expanded leaves (usually the fourth or fifth leaf from the apex) of two canes, randomly selected from the four central plants in each plot using a LI-COR 6200 portable photosynthesis system (LI-COR, Inc., Lincoln, Nebraska, USA) operated in closed system mode. CO_2 depletion by a 9.25 to 14.80 cm^2 leaf area in the leaf chamber was monitored for 15 to 60 seconds at air flow rates of 0.1 to 0.35 liter$\bullet min^{-1}$. All photosynthesis measurements were conducted at photosynthetic photon flux (PPF) greater than 1,000 $\mu mol \bullet m^{-2} \bullet s^{-1}$ and air temperatures ranged from 23 - 32C. The net CO_2 assimilation rates of floricane leaves were also determined at weekly intervals from just before anthesis until the beginning of harvest (1990). Two mature leaves from the middle of the flowering laterals and two middle laterals per floricane were used on two floricanes per plot randomly chosen from the centre part of each plot were used. Stomatal conductance, ambient and intercellular CO_2 concentrations, photosynthetic photon flux, ambient and leaf temperatures, air flow rates and relative humidity were also recorded and used as covariables in the analysis of covariance of net CO_2 assimilation rates. The net CO_2 assimilation rates of primocane and floricane leaves were not measured in 1991 since

no treatment effects were observed in 1989 and 1990.

Winter kill was assessed after bud break in May, 1990 and 1991 by examining the number of plants killed and the extent of cane die-back. Fruit yield and berry weight (mean of 25 fruit) were recorded at 3 - 4 day intervals on 4 central plants per plot from July 11 to August 8 in 1990 and from July 3 to July 25 in 1991.

A three-way analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) package (SAS Inc. 1985) and the means were separated by DMRT at the 5% level. The data derived from more than one observation date were subjected to ANOVA at each observation date and the homogeneity of pooled data were tested by Bartlett's test (Steel and Torrie, 1980). Significant interactions among cultivars, propagation methods and observation dates were found on all parameters examined. A fitted regression model between growth parameter and observation date was selected for each cultivar x propagation method combination using the stepwise procedure of SAS. The regression parameters of fitted regression models were compared for their similarity (Snedecor and Cochran, 1980).

3.4 Results and discussion

MP plants were less vulnerable than CP plants to field losses; 100% of MP but only 58% of CP plants survived field

establishment. Replacement of CP plants from the guard rows was necessary 45 days after field-planting. One hundred days after field-planting 100% of MP plants and 92% of CP plants (including the replants) survived. Survival figures remained the same in May 1990. Such high initial mortality among CP populations can be a serious problem in establishing new plantations. Propagation method did not affect winter hardiness which was not a problem despite the extreme low temperatures (-27 C) of January 1989.

Micropropagation promoted the vigor of 'Comet' and 'Festival' red raspberry plants compared with conventional propagation throughout this study as indicated by increased numbers and height of primocanes (Table 3.1, Fig. 3.1) and greater dry matter production (Table 3.2) in 1989 and 1990. The promotion of primocane numbers by MP was greater in 1990 than in 1989 for both cultivars and greater in 1991 for 'Festival' (Table 3.1). In 1990 primocane numbers were 100% and 300% greater in the MP than the CP plants for 'Comet' and 'Festival', respectively. In 1991 primocane numbers for MP 'Festival' were 50% greater than for CP 'Festival' but 'Comet' numbers were unaffected by propagation method. Primocane height was no longer affected by propagation method in 1991 (data not shown).

It is difficult to conclude whether the initial dormancy of CP canes affected establishment and depressed vigor relative to the MP plants. The MP plants certainly benefited

Table 3.1. The number of primocanes produced by each plant at the end of each growing season as affected by cultivar and propagation method. Each value is the mean of 48 plants.

Cultivars	Propagation method	Cane number 1989	1990	1991
Comet	CP	2.3 ab	11.0 b	21.8 a
Comet	MP	1.7 b	22.8 a	24.0 a
Festival	CP	2.0 b	6.7 c	15.0 b
Festival	MP	3.0 a	23.5 a	22.3 a

Mean separation in a column by DMRT at the 5% level.

Fig. 3.1. Primocane heights in 1989 and 1990 as affected by cultivar and propagation method. Each value is the mean of 48 canes.

Equations for 1989:

CP Comet: ● $Y=7.564-0.537X+0.013X^2-0.00005X^3$

$R^2=0.90$, $p<0.01$

MP Comet: ○ $Y=34.073-1.609X+0.043X^2-0.00001X^3$

$R^2=0.95$, $p<0.01$

CP Festival: ▲ $Y=6.976-0.593X+0.0148X^2-0.00007X^3$

$R^2=0.54$, $p<0.01$

MP Festival: △ $Y=15.912-1.194X+0.0323X^2-0.00016X^3$

$R^2=0.96$, $p<0.01$

Equations for 1990:

CP Comet: ● $Y=4.942+0.510X+0.016X^2-0.00007X^3$

$R^2=0.98$, $p<0.01$

MP Comet: ○ $Y=2.174+0.304X+0.017X^2-0.00007X^3$

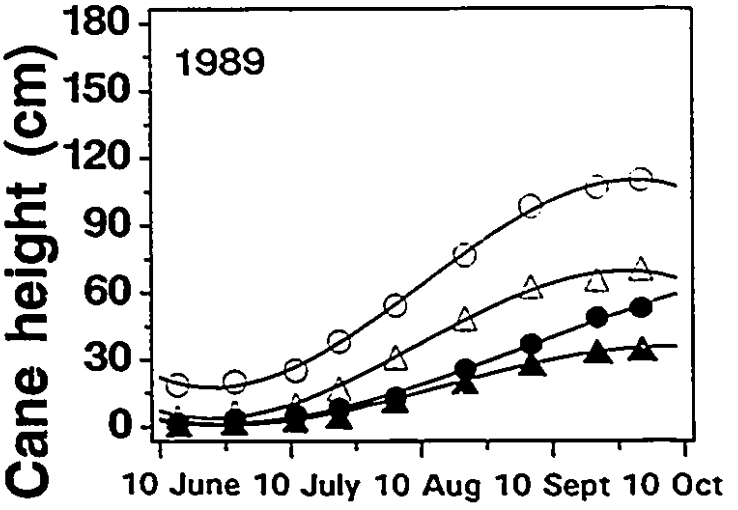
$R^2=0.99$, $p<0.01$

CP Festival: ▲ $Y=1.80+0.009X+0.019X^2-0.0001X^3$

$R^2=0.98$, $p<0.01$

MP Festival: △ $Y=2.139+0.285X+0.018X^2-0.0001X^3$

$R^2=0.99$, $p<0.01$


1989
Cane height (cm)
180
150
120
90
60
30
0
10 June 10 July 10 Aug 10 Sept 10 Oct

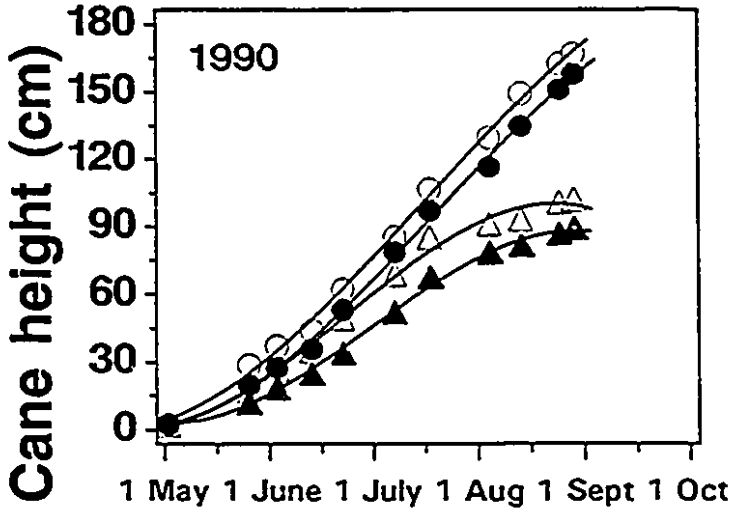

1990
Cane height (cm)
180
150
120
90
60
30
0
1 May 1 June 1 July 1 Aug 1 Sept 1 Oct

Table 3.2. The mean dry weights (g.plot^{-1}) of primocanes and floricanes removed from each plant at the end of the growing season in 1990 and 1991. Each value is the mean of 144 plants.

Propagation	Primocane		Floricane	
method	1990	1991	1990	1991
CP	81.4***	210.3**	22.3***	228.6**
MP	297.0	244.8	60.9	263.6

, * Mean separation within year by t test at P<=0.01, or 0.001, respectively.

from their faster initial growth rate with a carry-over effect into the second and third seasons. Increased vegetative vigor was also noted in blackberry (Swartz et al., 1983) and is a common effect of MP (Swartz, 1991). Cytokinin exposure in the multiplication medium may affect ex vitro growth performance (Swartz, 1991). Cane diameters are usually, but not always, positively correlated with fruit yields (Crandall et al., 1974a,b; Dale and Daubeny, 1985). MP plants produced thicker primocanes (7.12 mm) compared with CP plants (5.71 mm) in 1989 but, unfortunately, this trend did not persist.

No significant differences in CP and MP primocane and floricane appearance were noted in 1989 or 1990. In 1991 one instance of transient chlorotic leaves occurred on one MP 'Comet' cane. The etiology of this was unknown. Rosati et al. (1986) considered MP loganberry (*Rubus loganobaccus* Bailey) plants to be true-to-type and genetically stable after examination of 3698 MP plants in the field for 3 years. The risk of genetic variation is minimal when multiplication is done through axillary bud proliferation using pre-organized explants (shoot tips) and limiting the number of subcultures (Evans and Bravo, 1986). However, some epigenetic effects have been noted, such as the decreased herbicide (simazine and orysalin) tolerance of 'Heritage' red raspberry, limited to a short interval after field transplantation (Neal et al., 1990).

No differences in leaf photosynthetic rates of MP and CP

plants were observed although seasonal effects were apparent. The photosynthetic rates of primocanes increased early in the summer, decreased in mid summer and increased again later in the growing season (Fig. 3.2 A,B). The photosynthetic rates of floricanes were high at the beginning of anthesis (28 May to 2 June), declined as anthesis ended (15-20 June), increased during rapid berry development (20-30 June) and declined again as the berries began to ripen (early July) (Fig. 3.2 C). Photosynthetic comparisons are not available in the literature for MP and CP *Rubus* but higher photosynthetic rates were noted in MP 'Earliglow' but not in 'Redchief' strawberry (*Fragaria X ananassa* L.) compared with their CP counterparts (Cameron et al., 1989).

The total yields of MP 'Festival' during the first fruiting year were 303.7 $g \cdot plant^{-1}$, equivalent to 2.2 $MT \cdot ha^{-1}$, but were negligible from CP 'Festival' and CP and MP 'Comet' (Table 3.3). The fruit yield of MP 'Festival' reached 1157.4 $g \cdot plant^{-1}$, equivalent to 8.4 $MT \cdot ha^{-1}$, in 1991 and was the highest among all treatments. MP 'Festival' had greater numbers of fruit on longer laterals and larger fruit compared with CP 'Festival', although the fruit were not larger in 1991. However, propagation method did not affect yield of 'Comet' in either fruiting season. There is a report indicating that using strawberry MP plants directly for fruit production produced larger numbers of somewhat smaller fruit with less total yield

Fig. 3.2. The CO_2 uptake rates (mg $CO_2 \cdot dm^{-2} \cdot hr^{-1}$) of (A) primocane leaves in 1989 and (B) 1990, and (C) of floricane leaves in 1990 (C). Each value is the mean of 192 readings.

Equations for

A: $Y=13.10+0.10X-0.0046X^2+0.00003X^3$ $R^2=0.62$, $p<0.01$

B: $Y=11.90+0.18X-0.003X^2+0.00002X^3$ $R^2=0.50$, $p<0.01$

C: $Y=18.55-1.03X+0.05X^2-0.0008X^3$ $R^2=0.51$, $p<0.01$

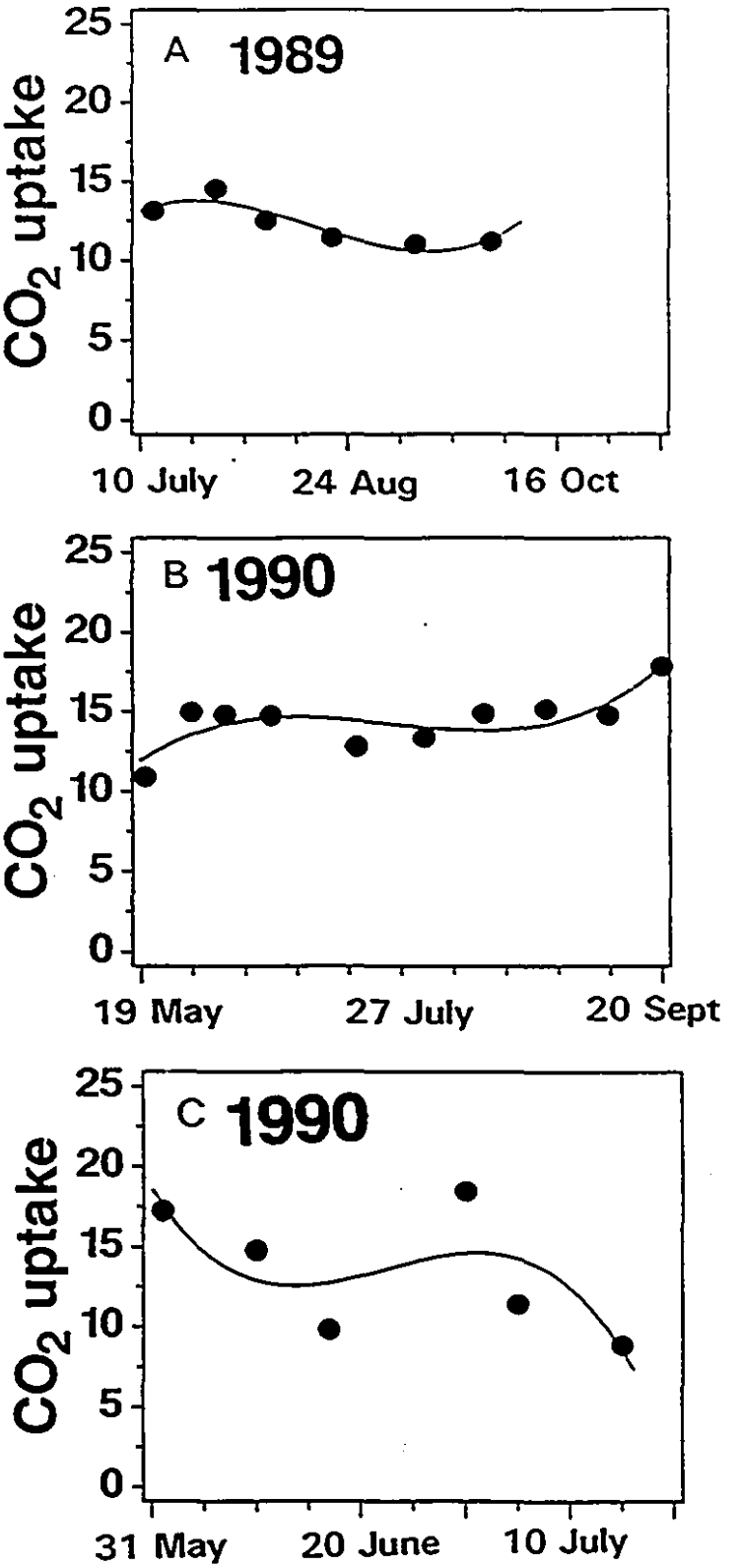

A 1989
B 1990
C 1990
CO_2 uptake
25
20
15
10
5
0
10 July
24 Aug
16 Oct
19 May
27 July
20 Sept
31 May
20 June
10 July

Table 3.3. The mean fruit yields of each plant in 1990 and 1991. Each value is the mean of 48 plants.

Cultivar	Propagation method	Fruit yield ($g \cdot plant^{-1}$) 1990	1991
Comet	CP	74.7 b	674.8 c
Comet	MP	85.1 b	787.1 bc
Festival	CP	62.1 b	934.8 b
Festival	MP	303.7 a	1157.4 a

Mean separation in a column by DMRT at 5% level.

compared with CP plants (Damiano et al., 1983). The assimilate allocation pattern in some red raspberry and strawberry cultivars may be altered by micropropagation in favour of fruit production (Cameron et al., 1989).

The effects of cane management system relative to propagation system were not observed on any of the parameters examined in our relatively young plantation regardless of cultivar and propagation method. Differences resulting from cane management systems were noted by Mason (1981) beginning in the fourth year after planting.

MP red raspberry plants showed promise as planting stock compared with bare rooted, dormant CP canes. The direct benefits of using MP plants are apparently better field establishment and greater initial growth and vigor. MP plants appear suitable for both nursery propagation and fruit production. Optimizing the cultural practices for MP plants may increase their benefit potential. Risk of variants appears to be negligible. To confirm the suitability of MP plants as planting stock further field comparisons should involve a greater number of cultivars, both floricane and primocane fruiting types, a greater number of test locations and CP material including both bare rooted canes and root cuttings from several nursery sources. The more widespread commercial scale use of MP red raspberry appears to be warranted in Quebec.

Preface to Chapter 4

Chapter 4 is a reproduction of a manuscript by R. Deng and D.J. Donnelly submitted to HortScience in November 1992. This chapter gives a comprehensive discussion of the in vitro hardening of red raspberry through CO_2 enrichment and medium sucrose reduction.

Chapter 4 In vitro hardening of red raspberry through CO_2 enrichment and medium sucrose reduction

4.1 Abstract

Micropropagated red raspberry (*Rubus idaeus* L. 'Festival') shoots were rooted in specially constructed plexiglass chambers under ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2 on media containing 0, 10, 20 or 30 $g \cdot liter^{-1}$ sucrose. Plantlet growth and leaf $^{14}CO_2$ fixation rates were evaluated prior to and 4 weeks after ex vitro transplantation. In vitro CO_2 enrichment promoted in vitro hardening; it increased both root number and length, plantlet fresh weight and photosynthetic capacity but did not affect other variables such as plantlet height, dry weight or leaf number and area. No residual effects of in vitro CO_2 enrichment were observed on 4-week-old transplants. Sucrose in the medium promoted plantlet growth but depressed photosynthesis and reduced in vitro hardening. Photoautotrophic plantlets were obtained on sucrose-free rooting medium under both ambient and enriched CO_2 conditions and they performed better ex vitro compared with mixotrophic plantlets grown on sucrose-containing media. Root hairs were more abundant and longer on root tips of photoautotrophic plantlets compared with mixotrophic plantlets. The maximum CO_2 uptake rates of plantlet leaves were 52% that of

greenhouse control plant leaves. This did not change in the persistent leaves up to 4 weeks after ex vitro transplantation. The photosynthetic ability of persistent and new leaves of 4-week-old ex vitro transplants related neither to in vitro CO_2 exposure levels nor to the medium sucrose concentrations. Consecutive new leaves of transplants exhibited gradually higher rates of CO_2 uptake than persistent leaves. The third new leaf of transplants had photosynthetic rates up to 90% that of greenhouse control plant leaves. The results indicated that in vitro CO_2 enrichment was beneficial to in vitro hardening and medium sucrose may be substantially reduced or eliminated from red raspberry rooting media when forced ventilation is used.

4.2 Introduction

Micropropagation has become an important technique for the commercial mass propagation of red raspberry (Donnelly and Daubeny, 1986; Zimmerman and Jones, 1991). In vitro red raspberry plantlets exhibit the phenotype typical of temperate species plants in vitro (the culture-induced phenotype or CIP) (Donnelly et al., 1985). This includes small thin leaves, reduced trichome numbers, reduced support tissues (collenchyma and sclerenchyma), increased percentage water content, permanently-open stomata and low photoautotrophic capacity. The CIP prevents micropropagates transferred directly from

culture to ambient greenhouse or field conditions from growing normally without special handling. Ex vitro transplants must undergo a period of acclimatization during which they are usually exposed to high relative humidity (RH) and relatively low light intensity for several days or weeks followed by a gradual adjustment of RH and light to ambient levels. During ex vitro acclimatization of *Rubus*, greenhouse- or field-grown (control) type anatomy and physiology were seen to develop gradually after a series of transitional organs (Donnelly and Vidaver, 1984a,b; Donnelly et al., 1984, 1985; Tisdall, 1989). The ex vitro acclimatization process can be expensive in terms of labor and controlled environment facilities. Manipulation of the culture environment to alter the CIP towards photoautotrophy and hardening could abbreviate or eliminate the ex vitro acclimatization period and reduce the overall costs of micropropagation (reviewed by Kozai, 1991; Preece and Sutter, 1991; Donnelly and Tisdall, 1992).

The photosynthetic rates of in vitro plantlets were very low perhaps limited by the low CO_2 levels in the culture vessels during the light period. CO_2 levels in culture vessels decreased to the CO_2 compensation point ($\leq$100 ppm) within 2 hours after the start of the light period and remained low throughout the light period (reviewed by Kozai, 1991). Improved growth and increased photosynthesis resulting from CO_2 enrichment in vitro have been demonstrated for several species, including carnation (*Dianthus caryophyllus*

L.) (Kozai and Iwanami, 1988), orchid (*Cybidium* spp.) (Kozai et al., 1987), potato (*Solanum tuberosum* L.) (Kozai et al., 1988), strawberry (*Fragaria x ananassa* Duch) (Desjardins, 1990; Kozai et al., 1991a), *Actinidia deliciosa* L. (Infante et al., 1989) and tobacco (*Nicotiana tabacum* L.) (Kozai et al., 1990b).

Sugar is an important element in the medium for most cultures; generally it promotes the growth of cultured plants. Shoot and root growth of jackfruit (*Artocarpus heterophyllus* Lam.) were increased linearly when the sucrose in the medium was increased from 0 to 20 $g \cdot liter^{-1}$ (Rahman and Blake, 1988). There was a positive relationship between the growth of in vitro rose (*Rosa multiflora* L.) plantlets and sugar concentration in the media up to 40 $g \cdot liter^{-1}$ (Langford and Wainwright, 1988). Similar responses occurred in potato shoots cultured on media with sucrose ranging from 0 to 30 $g \cdot liter^{-1}$ (Kozai et al., 1988). However, sucrose has adverse effects on the development of the photosynthetic apparatus and on the biosynthesis of chlorophyll (Van Huystee, 1977; Wetherell, 1982). There was a negative relationship between the CO_2-uptake-ability of plantlets and the medium sucrose concentration they were grown in. The CO_2 uptake rates of 'Iceberg' rose propagules grown on media containing 10, 20 or 40 $g \cdot liter^{-1}$ of sucrose were 20.77, 17.46 and 11.32 μmol CO_2 $h^{-1} \cdot g^{-1}$ fresh weight, respectively (Langford and Wainwright, 1988). The maximum net photosynthetic rates of rose plantlets

cultured on medium containing 1% sucrose were 10 times higher than that on medium containing 5% sucrose (Capellades et al., 1991). Inhibition of photosynthesis by sucrose in the medium is not completely understood. This might be related to the low rate of regeneration of the carboxylation substrate Ribulose-1,5-bisphosphate (RuBP) due to the accumulation of soluble sugars in the leaves and the inhibition of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity (Capellades et al., 1991; Grout and Donkin, 1987).

Several species have been cultured on sugar-free medium. For example, carnation (Kozai and Iwanami, 1988; Kozai et al., 1987), cauliflower (*Brassica oleracea L. var. botrytis*) (Grout and Donkin, 1987), Chinese mustard (*Brassica campestris* L.) (Kozai et al., 1991b), jackfruit (Rahman and Blake, 1988), orchid (Kozai et al., 1987), potato (Kozai et al., 1988a) and strawberry (Fujiwara et al., 1988; Kozai et al., 1991a) plantlets grew photoautotrophically on sucrose-free medium. In fact, autotrophy was promoted in cultured plantlets of carnation (Kozai and Iwanami, 1988), cauliflower (Grout and Donkin, 1987; Short et al., 1987), potato (Kozai et al., 1988a), rose (Langford and Wainwright, 1987; Capellades et al., 1991) and strawberry (Kozai et al., 1991a) on medium with reduced levels or without sucrose.

Most of the previous studies examined the effect of in vitro CO_2 and sucrose levels in the medium on plant growth separately; their relationship and their combined effects were

not understood. Although increasing in vitro CO_2 levels improved the growth and photosynthesis of several plant species, the optimal level of CO_2 has yet to be determined; it may be species and/or cultivar dependent. The benefits of reducing or omitting sucrose in the medium include promotion of autotrophy, cost-saving on materials and reduction of biological contamination. It is not known to what extent in vitro hardening can be achieved through CO_2 enrichment and medium sucrose reduction or omission.

The objectives of this study were to evaluate the effects of in vitro CO_2 at ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) levels and sucrose in the medium at 0, 10, 20 or 30 g•liter^{-1} on the growth and leaf $^{14}CO_2$ fixation of red raspberry plantlets prior to and after ex vitro transplantation. The appropriate criteria for in vitro hardened plantlets and ex vitro acclimatized transplants were also explored.

4.3 Materials and methods

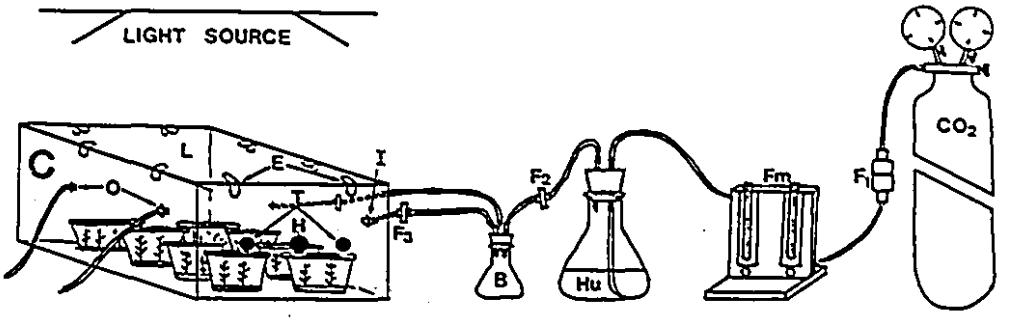
'Festival' red raspberry was micropropagated as previously described (Donnelly and Vidaver, 1984a). Shoots with 2 to 3 leaves and fresh weights of 23 ± 4 mg were rooted under either ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2 conditions on Murashige and Skoog (1962) basal salt medium with either 0, 10, 20, or 30 g•liter^{-1} sucrose supplemented with 1.2 μM thiamine·HCl, 550 μM inositol, 2.45

μM indole-3-butanoic acid (IBA) and 5.5 g•liter^{-1} agar (Anachemia) adjusted to pH 5.7.

The experiment was carried out in specially designed sterile plexiglass incubation chambers (55 x 30 x 15 cm) with tightly-fitting plexiglass lids, secured with elastic bands wrapped around paired hooks (Fig. 4.1).. The pre-mixed and analyzed gas mixtures of either ambient or enriched CO_2 from compressed cylinders were continuously supplied to the chambers at a flow rate of 15 ml•min^{-1} through a flow meter and scrubbed with a series of filters before they were humidified in a 4.5 L erlenmeyer and buffered in small erlenmeyer flasks that preceded the incubation chambers. Each chamber had two air inlets and two air outlets on the opposite side and three sensor probe ports; two for temperature and one for humidity. The chambers and tubing systems were surface sterilized with 10% bleach and 70% ethanol, respectively. The temperatures inside the chambers were monitored at intervals through probe-ports in the plexiglass chambers with a telethermocouple (Cole-Parmer, Model 8500-40, Chicago, Il). The 2 levels of CO_2 and 4 levels of medium sucrose treatment combinations were arranged as a 2 x 4 factorial experiment according to a split plot design with CO_2 as the main plot factor and medium sucrose as the subplot factor with 4 replicates. Each replicate consisted of one container with 9 shoots.

After 4 weeks of incubation, 2 plantlets from each replicate were destructively harvested to determine their root

Fig. 4.1. The experimental set up showing the plexiglass incubation chambers and the CO_2 control systems. Tight fitting plexiglass lids were secured to the chambers (C) with elastic bands (E) wrapped around paired hooks. CO_2 gas mixtures travelled from compressed gas cylinders through a flow meter (Fm) and a series of filters (F1 = in line, type 407, ADC; F2 = syringe type, 0.4 μm; and F3 = syringe type, 0.2 μm) and were humidified in a large erlenmeyer (Hu) before entering a buffering chamber (B) which preceded the incubation chambers. Air entered each incubation chamber through two air inlets (I) and flushed out through two air outlets (O) on the opposite side. The temperature and RH in the chambers were monitored through the two temperature probe ports (T) and one RH probe port (H).


LIGHT SOURCE
C
L
E
I
O
T
H
F3
F2
B
Hu
Fm
F1
CO2

numbers, total root lengths, leaf numbers, total leaf areas, heights and fresh and dry weights. Five additional plantlets, each with its youngest leaf tagged, were transferred to cell packs containing a substrate of 2 Promix (BX) : 1 loam soil and incubated in a growth chamber at 25C under cool white fluorescent light of 120 ± 5 $\mu Mol \cdot m^{-2} \cdot s^{-1}$ (400 - 700 nm) and 16 hour photoperiod for 4 weeks. The cell packs were covered with transparent plastic covers for 10 days to maintain a high RH. Two 4-week-old transplants from each replicate were sampled for growth evaluation. Dry weights were obtained after drying the plant tissues in an oven at 70C for at least 48 hours until constant weight. Five root tips of about 1 cm long were excised from each replicate and stained with aqueous safranin (1%) for 1 min before examining with a light microscope.

Two plantlets, two 4-week-old ex vitro transplants from each replicate and five control leaves from 1-year-old, tissue culture derived, greenhouse-grown plants were subjected to $^{14}CO_2$ fixation assay modified from Donnelly et al. (1984). The labeling apparatus consisted of a closed gas circuit with a sample chamber (15 cm^3) to which light was supplied at 100 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400 - 700 nm) by cool white fluorescent lights. Each plant was exposed to the $^{14}CO_2$ (370 KBq) gas mixture for 5 min ± 10 s. Excess $^{14}CO_2$ was absorbed in 200 ml 2N NaOH at the end of each exposure. After exposure, leaf discs (29.5 mm^2) excluding the major veins were taken from each leaf using

a paper punch. The labeled tissues were placed in 80% ethanol within 5 seconds and soaked for 2 - 3 days to extract the ^{14}C-labeled compounds (Deng and Donnelly, 1992a). After extraction, two 100 μl subsamples were used for determination of ^{14}C activity in 5 ml liquid scintillation cocktail (Universol, ICN Biochemicals Inc.,) in a Beckman LS-5801 liquid scintillation spectrometer. The ^{14}C activity was measured in the 0-670 energy window, at an efficiency of 90-95% (H# between 60 to 80), corrected for background activity and adjusted for ethanol extract volume. The specific activity of labeled tissues was expressed as 1,000 dpm (Kdpm) per cm^2.

Analysis of variance was performed using the General Linear Model procedure (GLM) of the Statistical Analysis System (SAS) (SAS Inc, 1985) on the means of each experimental unit. Homogeneity of variance was tested using the Bartlett's test (Steel and Torrie, 1980) and appropriate transformations were used where necessary (Gomez and Gomez, 1984).

4.4 Results

All plantlets survived the 4 week in vitro incubation period regardless of the CO_2 or medium sucrose levels. Both CO_2 and medium sucrose affected the growth of in vitro plantlets independently. In vitro CO_2 enrichment significantly increased plantlet root number, total root length and total fresh weight compared with those grown under

ambient CO_2 (Table 4.1). Plantlets grown under enriched CO_2 also had a healthier appearance compared with those grown under ambient CO_2. However, other parameters such as plantlet height, percentage dry weight, leaf number and area were similar among plantlets grown under both CO_2 levels (data not shown).

Plantlets grew successfully on sucrose-free medium suggesting that photoautotrophy was established. These photoautotrophic plantlets had shorter total root length (3.2 ± 0.6 cm) and lighter fresh weights (225 ± 16 mg) compared with the mixotrophic plantlets that were grown in sucrose-containing media (7.3 ± 0.9 cm and 337 ± 25 mg, respectively). Plantlet fresh weight, but no other parameter increased with increasing concentration of medium sucrose (Fig. 4.2). Although root hairs were present on all root tips examined, those on root tips of plantlets grown on sucrose-free medium were significantly longer and consistently stained darker than those on root tips of plantlets grown on sucrose-containing media regardless of the amount of sucrose in the media (Fig. 4.3). The root hair zone on root tips of plantlets grown on sucrose-free medium began closer to the tip and hairs were more abundant compared with those of plantlets grown on sucrose-containing media.

The ^{14}C activity levels in cultured plantlet leaves were negatively related to the sucrose concentrations in the medium under enriched but not ambient CO_2 conditions (Fig. 4.4). The

Table 4.1. The effects of ambient or enriched CO_2 levels in vitro on the root number and length and fresh weight of red raspberry plantlets.

CO_2 levels	Root		Fresh
	Number	Length (cm)	weight (mg)
(ppm)			
340	4.9	4.4	276
1500	7.3	8.2	346
Sig.[a]	**	**	**

[a] ** significantly at $p<0.01$.

Fig. 4.2. Micrograph of representative root tips of (A) photoautotrophic plantlets showing abundant and long root hairs and (B) mixotrophic plantlets showing fewer and shorter root hairs. Bar = 0.5 mm.

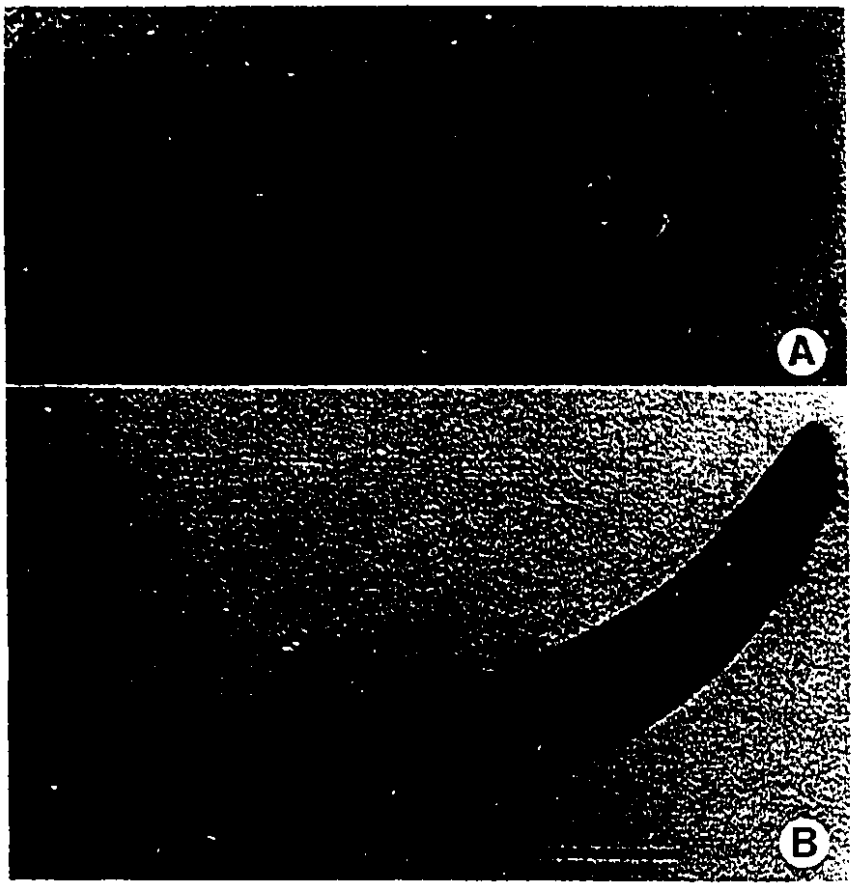

A
B

Fig. 4.3. Effects of medium sucrose concentration on total plantlet fresh weight after 4 weeks in culture. Values are the means of 48 samples.

Regression equation:

$Y=236.6+4.83X \quad R^2=0.42 \quad p<0.01$

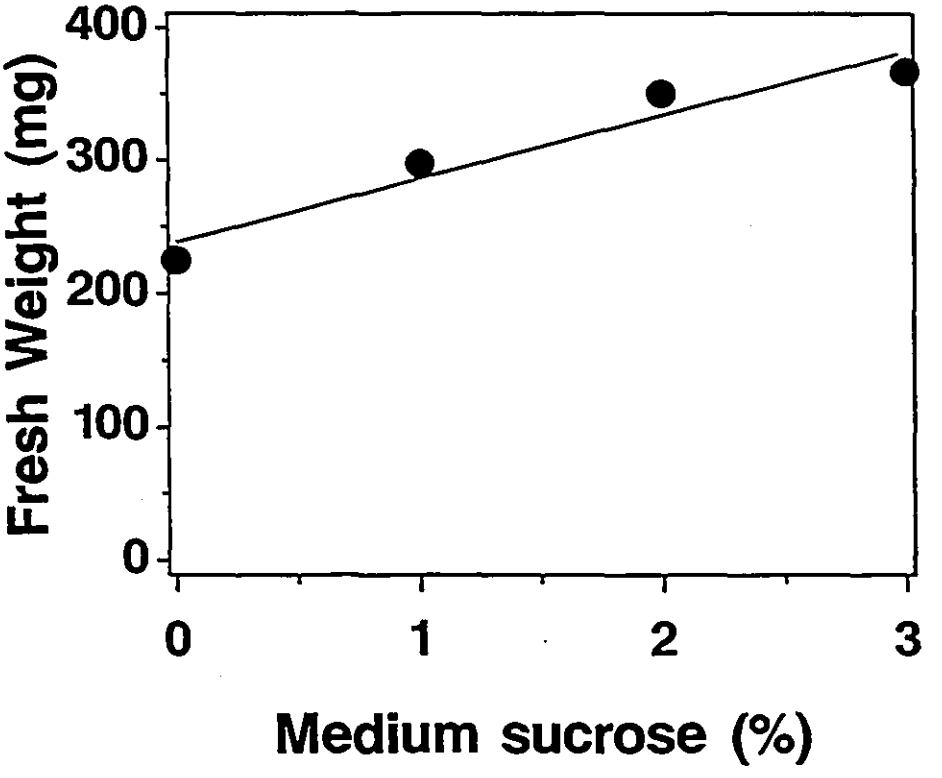

Fresh Weight (mg)
400
300
200
100
0
0
1
2
3
Medium sucrose (%)

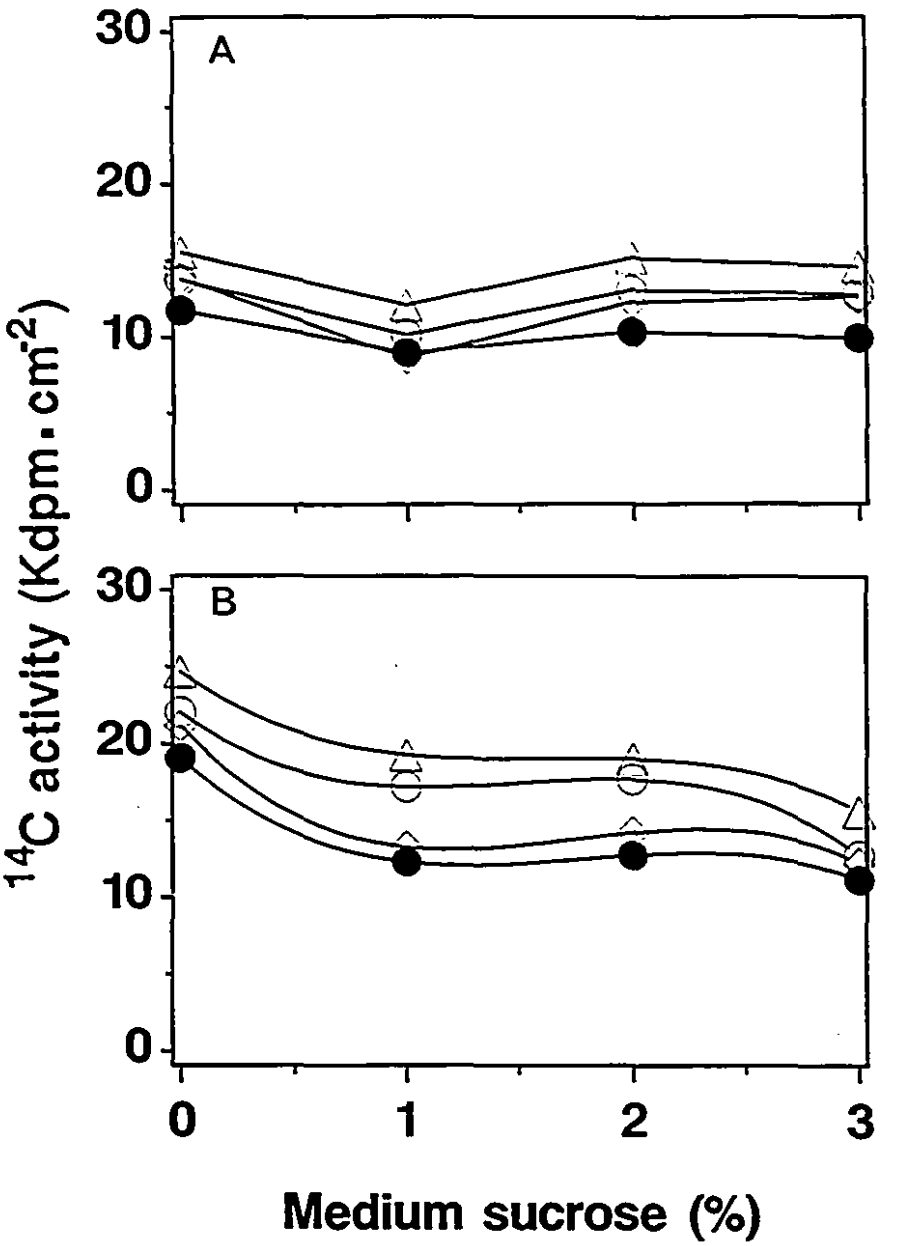
Fig. 4.4. Effects of CO_2 at (A) ambient or (B) enriched levels and medium sucrose concentrations from 0 to 3% on $^{14}CO_2$ activity levels in plantlet leaves (numbered basipetally). Value is the mean of 12 samples.

Leaf No. 1: * $Y=19.10-1.340X+0.082X^2-0.0015X^3$ $R^2=0.88$ $p<0.01$

Leaf No. 2: o $Y=22.03-1.105X+0.081X^2-0.0018X^3$ $R^2=0.71$ $p<0.01$

Leaf No. 3: Δ $Y=24.69-1.078X+0.068X^2-0.0014X^3$ $R^2=0.70$ $p<0.01$

Leaf No. 4: ◇ $Y=21.15-1.615X+0.102X^2-0.0020X^3$ $R^2=0.64$ $p<0.01$


A
B
30
20
10
0
0
1
2
3
^{14}C activity (Kdpm · cm^{-2})
Medium sucrose (%)

mean ^{14}C activity levels in cultured plantlet leaves grown under enriched CO_2 were higher (16.7 ± 1.4 Kdpm•cm^{-2}) compared with plantlets grown under ambient CO_2 conditions (12.3 ± 0.8 Kdpm•cm^{-2}) indicating a higher photosynthetic capacity.

Four weeks after ex vitro transplantation, transplants from photoautotrophic plantlets had developed significantly larger leaf areas (4.1 ± 0.4 cm^2) and greater fresh weights (983 ± 76 mg) compared with transplants from mixotrophic plantlets (2.6 ± 0.2 cm^2 and 643 ± 59 mg, respectively). However, all other growth parameters, i.e. root number, total root length, leaf number (persistent and new leaves) and transplant height were similar in all transplants (data not shown).

The ^{14}C activity levels in persistent leaves of 4-week-old transplants were not correlated to in vitro CO_2 levels or medium sucrose concentrations (data not shown). The ^{14}C activity in persistent leaves of 4-week-old transplants was obtained from tagged leaves which had completed 10 - 50% of their final expansion at the time of transplantation. The overall average ^{14}C activity levels in these persistent leaves were similar to that of plantlet leaves or about 53% that of control plant leaves (Table 4.2). The CO_2 uptake ability of new leaves formed on 4-week-old transplants was not correlated to in vitro CO_2 levels or medium sucrose concentrations (data not shown). Consecutively developed new leaves had gradually higher $^{14}CO_2$ uptake capacity than the persistent leaves (Table

Table 4.2. The ^{14}C activity levels of plantlet leaves, persistent and new leaves of ex vitro transplants and greenhouse control plant leaves after the plants were exposed to 10 370 KBq $^{14}CO_2$ for 5 min. Values represent the mean of 64 leaves except for control values which represent the mean of 5 leaves.

Ex vitro time(wk)	Leaves	Activity (Kdpm•cm^{-2})	Percentage of control
0	Plantlet	14.5 ± 1.3	51.8
4	persistent	14.8 ± 1.1	52.9
	New 1	14.5 ± 1.6	51.8
	New 2	22.2 ± 2.0	79.3
	New 3	25.0 ± 1.6	89.3
	Control	28.0 ± 1.2	100

4.2). The $^{14}CO_2$ uptake ability of the third ex vitro developed leaf reached 90% that of control leaves (Table 4.2).

4.5 Discussion

The reported photosynthetic capacities of cultured plantlets were low, ranging from negative or zero (net respirers) in photosynthetically incompetence species such as cauliflower (Grout and Aston, 1978a) and strawberry (Grout and Millam, 1985), to slightly positive (<5 mg CO_2 $dm^{-2} \cdot hr^{-1}$) in photosynthetically competent species such as red raspberry (Donnelly and Vidaver, 1984b), *Actinidia deliciosa* (Infante et al., 1989), tobacco (Kozai et al., 1990b), orchid (Kozai et al., 1990a) and Asian white birch (Smith et al., 1986) when measured under higher light intensity than levels commonly used in vitro. The low photosynthetic capacities of cultured plantlets was attributed mainly to the low CO_2 levels inside the culture vessels (reviewed by Kozai, 1991).

In this study, gas exchange was improved through open vessels and by forced ventilation to incubation chambers. In vitro enriched CO_2 promoted root formation and increased the fresh weight of red raspberry, supporting the observations on strawberry (Kozai et al., 1991a), tobacco (Kozai et al., 1990b), carnation (Kozai and Iwanami, 1988), orchid (Kozai et al., 1987) and potato (Kozai et al., 1988a). Increased growth seemed to relate to relatively high photosynthetic ability.

The CO_2 uptake rates of red raspberry plantlets were higher under enriched than under ambient CO_2 conditions confirming that CO_2 levels were indeed limiting during conventional micropropagation. Improved photosynthesis corresponding to higher vessel CO_2 levels was also observed in cultured plantlets of strawberry (Desjardins, 1990; Kozai et al., 1991a) and *Actinidia deliciosa* (Infante et al., 1989).

The effects of in vitro CO_2 enrichment of red raspberry did not carry over to 4 weeks ex vitro, unlike results with strawberry (Desjardins, 1990). Improved transplant growth using ex vitro CO_2 enrichment has been reported for grape (Lakso et al., 1986) and strawberry (Desjardins et al., 1987). The potential benefits of CO_2 enrichment are probably best achieved by extending this treatment ex vitro.

Sugars were essential in culture medium for many species and in some cases independent growth could not be achieved on medium without sucrose during proliferation (Langford and Wainwright, 1987) or during rooting (Grout and Price, 1987). However, it was found that strawberry (Kozai et al., 1991a) and carnation (Kozai and Iwanami, 1988) plantlets rooted well on sucrose-free medium. In fact photosynthesis was higher in plantlets cultured on sucrose-free medium than those on sucrose-containing medium, i.e. cauliflower (Grout and Donkin, 1987), rose (Capellades et al., 1991; Langford and Wainwright, 1987) and potato (Kozai et al., 1988a). Our results also confirm this (Fig. 4.4).

Root function is positively linked to the number and length of root hairs on the root tips (Nobel, 1991). The enormous number of long root hairs on the root tips of photoautotrophic plantlets would tend to improve root function after transplantation. Photoautotrophic plantlets performed better than mixotrophic plantlets . after ex vitro transplantation for up to 4 weeks despite their shorter roots and lower fresh weights at the time of transplantation. It was clear that sucrose was responsible for the root hair differences. However, the relationship between the number and length of root hairs on root tips and the levels of sucrose in the medium was not understood. It was not correlated to the differences in water potential of the media since root tips were similar on plantlets grown on medium containing 10, 20 or 30 $g \cdot liter^{-1}$ sucrose.

Carbon dioxide uptake measured by infra-red gas analysis (IRGA) showed that red raspberry plantlet leaves had CO_2 uptake rates of only 2 - 3 $mg \cdot dm^{-2} \cdot h^{-1}$ or about 20% that of control plant leaves (10 - 15 $mg \cdot dm^{-2} \cdot h^{-1}$) (Donnelly and Vidaver, 1984b). While this was considerably less than the apparent 62% of control leaves indicated by $^{14}CO_2$ labeling methods (Donnelly et al., 1984), the $^{14}CO_2$ technique usually yields CO_2 uptake rates 2 - 3 times higher than the IRGA method (Karlsson and Sveinbjornsson, 1981). In this study, the $^{14}CO_2$ uptake rates of plantlet leaves were 52% of control plant rates and did not change up to 4 weeks ex vitro (Table

4.2). Red raspberry persistent leaves could last up to 3 months ex vitro during conventional acclimatization (Donnelly and Vidaver, 1984a). These results indicated that the potential photosynthetic contribution of leaves of plantlets and persistent leaves of transplants was relatively high.

Consecutively developed new leaves had gradually higher CO_2 uptake rates than the persistent leaves. Similarly, leaves with transitional phenotype were observed in *Leucaena leucocephala* (Lam) De Wit (Dhawan and Bhojwani, 1987), strawberry (Fabbri et al., 1986), cherry (*Prunus cerasus* L.) (Marin et al., 1988) and sweetgum (*Liquidambar styraciflua* L.) (Wetzstein and Sommer, 1982). At least 5 transitional leaves were produced on red raspberry transplants from conventional micropropagation during conventional ex vitro acclimatization (Donnelly et al., 1984). We found that the CO_2 uptake rates of the third ex vitro new leaves reached 90% that of control leaves.

Plantlets may be considered hardened if they can survive ex vitro ambient conditions with minimal or no extra precautions when transferred ex vitro; they must have relatively high photosynthetic capacity and narrow stomatal apertures. Hardened plantlets may still show many aspects of the CIP and will produce transitional leaves after transplantation. Transplants may be considered fully acclimatized if they have functional stomata and photosynthetic capacity comparable to that of control plants.

When acclimatization is completed transitional leaves are no longer formed; leaves with the control-type phenotype are produced.

The number of transitional leaves formed on transplants appears to depend on the degree of hardening of the cultured plantlets and the stress of the new ex vitro environment. The fewer the number of transitional leaves, the briefer the acclimatization interval. The reduced number of transitional leaves produced on transplants in the current study compared with conventional micropropagates suggested that the cultured plantlets were hardened to a great extent. This was probably due to growth under forced ventilation.

Photoautotrophic red raspberry plantlets were established on sucrose-free medium under either ambient or enriched CO_2 conditions, and their ex vitro performance was superior to that of mixotrophic plantlets. Red raspberry could be rooted on medium without or with reduced sucrose when forced ventilation was used. In vitro CO_2 enrichment improved plantlet quality and photosynthetic ability, enhanced ex vitro performance and reduced the ex vitro acclimatization interval.

Preface to Chapter 5

Chapter 5 is a reproduction of a manuscript by R. Deng and D.J. Donnelly which was presented orally by R. Deng at the 1992 World Congress on Cell and Tissue Culture held in Washington, D.C., U.S.A. from June 20 to June 25, 1992. The manuscript was submitted to Plant Cell, Tissue and Organ Culture in November 1992. This chapter gives a comprehensive discussion of the in vitro hardening of red raspberry by CO_2 enrichment and relative humidity reduction on low-sucrose medium.

Chapter 5 In vitro hardening of red raspberry by CO_2 enrichment and relative humidity reduction on low-sucrose medium

5.1 Abstract

The growth, $^{14}CO_2$ fixation and stomatal function of cultured red raspberry plantlets, rooted in specially designed incubation chambers under ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2 and ambient (100%) or reduced (90%) relative humidity (RH) on medium containing 0, 1 or 5 g•liter^{-1} sucrose, were evaluated prior to and 2 and 4 weeks after ex vitro transplantation. The objective was to harden plantlets in vitro. In vitro CO_2 enrichment increased the growth and general vigor, root formation and CO_2 uptake rate of plantlets and improved ex vitro transplant performance. Stomatal index and apertures on leaves of cultured plantlets were increased under CO_2 enrichment. However, this did not affect the ex vitro performance of transplants. In vitro RH of 90% decreased plantlet growth, narrowed stomatal apertures and improved plantlet ex vitro performance but did not affect the CO_2 uptake rates of cultured plantlets or ex vitro transplants. Photoautotrophic plantlets were obtained on sucrose-free medium. Medium sucrose promoted plantlet growth but reduced their CO_2 uptake rates. The stomata on cultured plantlet leaves were not functional and remained so up to 4

weeks after ex vitro transplantation. Normal stomatal function occurred on the second new leaves formed ex vitro. Cultured plantlet leaves had CO_2 uptake rates 69% that of control plant leaves which declined in the persistent leaves to 56% by 4 weeks ex vitro. Consecutively developed new leaves of ex vitro transplants had gradually higher CO_2 uptake rates than the cultured plantlet leaves and the persistent leaves; the fourth new leaves reached greenhouse control-plant levels. Completely in vitro hardened plantlets were obtained under in vitro CO_2 enriched and reduced RH treatments regardless of the amount of sucrose in the medium. These hardened plantlets were successfully transferred directly to soil under ambient greenhouse conditions without any special precautions.

5.2 Introduction

Conventional micropropagates posses a cultured-induced phenotype (CIP) with anatomical and physiological characteristics different from those of greenhouse- or field-grown plants such as small thin leaves, reduced trichome numbers, decreased foliar waxes, reduced support tissue, increased water content, permanently-open stomata and low photoautotrophic capacity (reviewed by Donnelly & Tisdall, 1992; Preece & Sutter, 1991; Ziv, 1991). When cultured plantlets are transferred to ex vitro conditions they must

usually be kept under high humidity (96 - 100%) and relatively low light intensity for a period of 1 month or more until they are acclimatized to ambient conditions. The control-type phenotype develops gradually through a series of transitional organs during ex vitro acclimatization. Commercially, the ex vitro acclimatization step is expensive due to the requirements for tenting or misting facilities and associated intensive labor (Debergh, 1988).

The CIP reflects acclimatization to the in vitro environment, typically characterized by limited gas exchange to culture vessels, saturated atmosphere (ca. 100% relative humidity, RH) and exogenous sugars and plant growth regulators. Modification of the in vitro environment to resemble external ambient conditions can promote in vitro hardening and reduce the ex vitro acclimatization interval. Improved growth and increased photosynthetic capacities at higher in vitro CO_2 levels have been observed in cultured strawberry (*Fragaria x ananassa* Duch.) (Desjardins, 1990; Kozai et al., 1991a), tobacco (*Nicotiana tabacum* L.) (Kozai et al., 1990c), carnation (*Dianthus caryophyllus* L.) (Kozai & Iwanami, 1988), orchid (*Cymbidium* spp.) (Kozai et al., 1987) and potato (*Solanum tuberosum* L.) (Kozai et al., 1988a). Lower in vitro RH promoted hardening of cultured rose (*Rosa multiflora* L.) (Capellades et al., 1990), chrysanthemum (*Chrysanthemum moriffolium* L.) (Short et al., 1987) and carnation (Ziv et al., 1983). Although sucrose is an

important component of the culture medium for most species; carnation (Kozai & Iwanami, 1988), cauliflower (*Brassica oleracea* L.) (Grout & Donkin, 1987; Short et al., 1987), potato (Kozai et al., 1988a), rose (Langford & Wainwright, 1987; Capellades et al., 1991) and strawberry (Kozai et al., 1991a) could be cultured photoautotrophically on sucrose-free medium. Red raspberry (*Rubus idaeus* L.) plantlets could also grow photoautotrophically on sucrose-free medium (Deng and Donnelly, 1992). However, their growth was reduced compared with plantlets grown on sucrose-containing media. Red raspberry plantlets may require some sucrose in the medium to attain maximum growth.

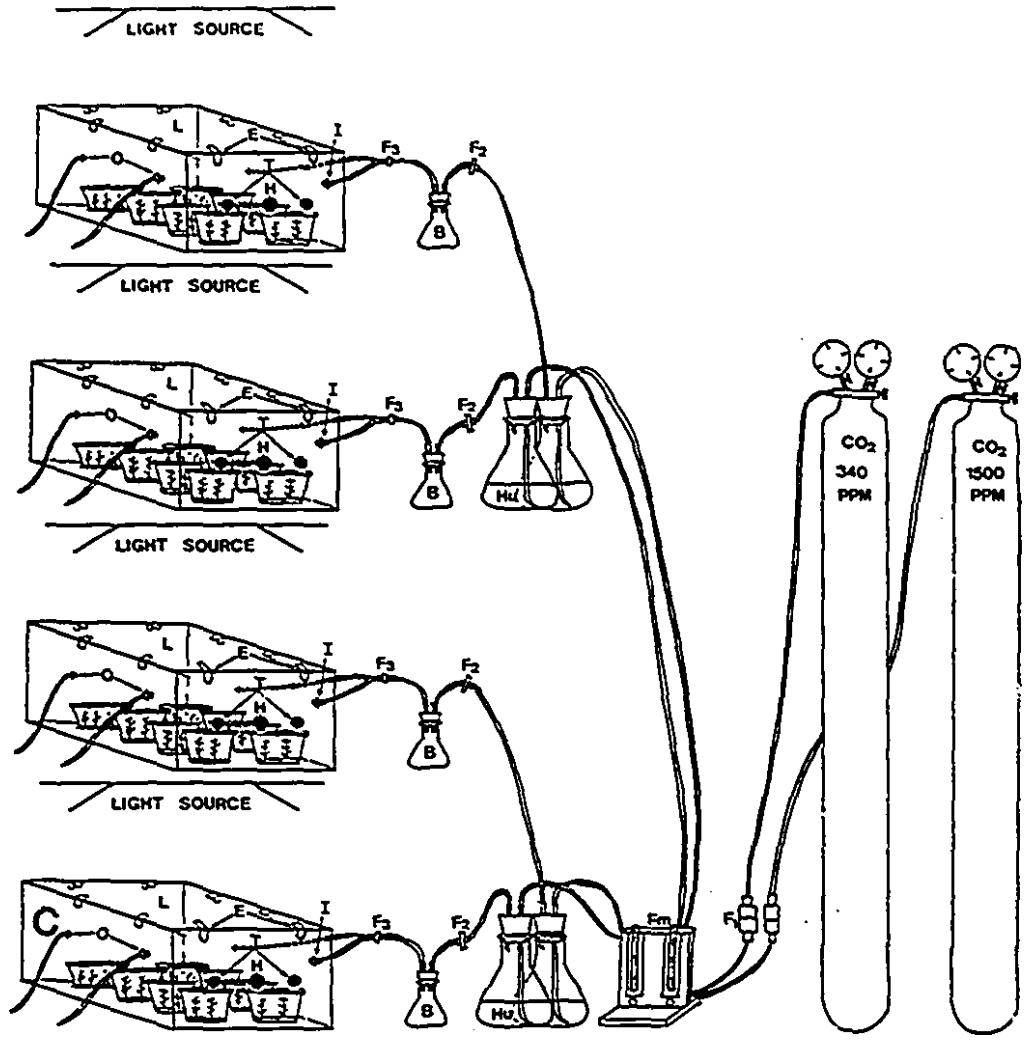
This study examined the effects of in vitro ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2, ambient (100%) or reduced (90%) RH and low levels of sucrose in the media (0, 1 or 5 g•liter^{-1}) on the growth, $^{14}CO_2$ fixation and stomatal function of cultured red raspberry plantlets and ex vitro transplants. The objective was to harden red raspberry plantlets in vitro so that they could be transferred to soil without special precautions.

5.3 Materials and methods

'Comet' red raspberry was micropropagated as previously described (Donnelly and Vidaver 1984a). Shoots with 2 to 3 leaves and fresh weights of 23 ± 4 mg were rooted for 4 weeks

under ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2 and ambient (100%) or reduced (90 ± 5%) RH conditions. Medium consisted of Murashige and Skoog (1962) basal salt with either 0, 1 or 5 g•liter^{-1} sucrose supplemented with 1.2 μM thiamine·HCl, 550 μM inositol, 2.45 μM indole-3-butanoic acid (IBA) and 5.5 g•liter^{-1} agar (Anachemia) adjusted to pH 5.7. The 2 levels of CO_2, 2 levels of RH and 3 levels of sucrose were arranged as a 2 x 2 x 3 factorial experiment according to a split plot design with CO_2 and RH treatment combinations as the main plot factors and medium sucrose as the subplot factor. Each of four replicates consisted of two open magenta containers with nine shoots each. The experiment was carried out in sterile plexiglass incubation chambers (Fig. 5.1). Continuous streams of CO_2 gas mixtures from compressed cylinders were regulated by a flow meter to 15 ml•min^{-1} and scrubbed with a series of filters before they were humidified in a 4.5 L erlenmeyer and buffered in a 250 ml erlenmeyer flask before entering the incubation chambers. Each chamber had two air inlets and two air outlets on the opposite side and three sensor probe ports; two for temperature and one for humidity. The chambers and tubing systems were surface sterilized with 10% bleach and 70% ethanol, respectively. Chamber RH of either 100% or 90% was maintained by bubbling the gas mixtures through a humidifier with 2 L of either pure water or a solution of 45% glycerol (w/v) (modified from Forney and Brandl, 1992) in 4.5 L erlenmeyer flasks. The RH

Fig. 5.1. The experimental set up showing the CO_2 and RH control systems and the plexiglass incubation chambers. Tight fitting plexiglass lids were secured to the chambers (C) with elastic bands (E) wrapped around paired hooks. CO_2 gas mixtures travelled from compressed gas cylinders through a flow meter (Fm) and a series of filters (F1 = in line, type 407, ADC; F2 = syringe type, 0.4 μm; and F3 = syringe type, 0.2 μm) and were humidified in a large erlenmeyer (Hu) before entering a buffering chamber (B) which preceded the incubation chambers. Air entered each incubation chamber through two air inlets (I) and flushed out through two air outlets (O) on the opposite side. The temperature and RH in the chambers were monitored through the two temperature probe ports (T) and one RH probe port (H).


LIGHT SOURCE
L
E
I
T
H
F3
F2
B
LIGHT SOURCE
L
E
I
T
H
F3
F2
B
Hu
LIGHT SOURCE
L
E
I
T
H
F3
F2
B
LIGHT SOURCE
C
L
E
I
T
H
F3
F2
B
Hu
Fm
CO2
340
PPM
CO2
1500
PPM

and temperature inside the chambers were monitored twice weekly through probe-ports in the plexiglass chambers with a Humicor, IHRT digital RH/Temperature indicator and a telethermocouple (Cole-Parmer, Model 8500-40, Chicago, Il). The cultures were maintained at 25 ± 3C with a light intensity at plant height of 55 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (cool white fluorescent) and a 16 hour photoperiod.

After 4 weeks of incubation, all experimental units were subjected to initial evaluation of numbers of surviving shoots, rooted shoots and a subjective grading of general shoot vigor (1 - 5; 1 being the least vigorous). Three plantlets were randomly selected from each experimental unit and destructively harvested to determine their heights, number of leaves and roots, total root lengths, fresh weights, dry weights and leaf areas. For dry weights, plantlets were dried in an oven at 70C for 48 hours until constant weight. Stomatal index (S.I.), apertures and numbers of trichomes were determined on 8 leaves from each experimental unit. Four leaves were randomly selected at 5 hours into the light period and 4 leaves at 5 hours into the dark period. The detached leaves were fixed in 80% ethanol within 3 sec of excision to prevent changes in stomatal aperture. Cleared leaves (Tisdall and Donnelly 1988) were examined with a Leco 2001 image analyzer (Leco, Montreal, Canada). Five fields, each with about 60 epidermal cells and 10 - 14 stomata, were randomly chosen on each leaf to determine the numbers of epidermal

cells and stomata and the stomatal apertures. Stomata with apertures less than 2.0 μm were considered closed.

Two plantlets, randomly selected from each experimental unit, were subjected to $^{14}CO_2$ fixation assay modified from Donnelly et al. (1984). The labeling apparatus consisted of a closed gas circuit with a plexiglass sample chamber (15 cm^3) to which light was supplied at 100 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400 - 700 nm) by 3 cool white fluorescent lights. The gas circuit included a gas generator, gas mixing chamber, CO_2 trapping flask and a vacuum pump. Five hundred μl of $Na_2{}^{14}CO_3$ solution (total activity: 20 μCi) was placed in the gas generator receptacle and 6 ml of 1N HCl was injected through the rubber stopper into the receptacle using a syringe to release the $^{14}CO_2$ into the gas circuit excluding the sample chamber. To equilibrate the $^{14}CO_2$ the gas was circulated through the gas circuit by a vacuum pump (MasterFlex, Cole Parmer instrument Inc., U.S.A.) at 350 $ml \cdot min^{-1}$ for 10 min excluding the sample chamber. Each plantlet was exposed to $^{14}CO_2$ gas mixture for 5 min ± 10 s. Excess $^{14}CO_2$ was absorbed in 200 ml 2N NaOH at the end of each exposure. The samples were removed from the chamber and leaf discs (29.5 mm^2), excluding the major veins, were taken from each leaf using a paper punch. The labeled tissues were placed in 80% ethanol within 5 seconds and kept for 2 to 3 days to extract the ^{14}C-labeled compounds. After extraction, two 100 μl subsamples were withdrawn for ^{14}C activity determination in 5 ml liquid scintillation cocktail

(Universol, ICN Biochemicals Inc.,) with a Beckman LS-5801 liquid scintillation spectrometer. The ^{14}C activity was measured in the 0 - 670 energy window, at an efficiency of 90 - 95% (H# between 60 to 80), corrected for background activity and adjusted for ethanol extract volume. The specific activity of labeled tissue was expressed as 1,000 dpm (Kdpm) per cm^2. The control plant leaves were from 1-year-old, tissue culture-derived greenhouse-grown plants.

The remaining plantlets, each with its youngest leaf tagged, were transferred to cell packs containing a substrate of 2 Promix (BX) : 1 loam soil and incubated in a growth chamber at 30 - 40% RH, 25 ± 2C under cool white fluorescent light of 120 ± 5 $\mu Mol \cdot m^2 \cdot s^{-1}$ (400 - 700 nm) and 16 hour photoperiod for 4 weeks. All transplants were exposed to ambient conditions since no covers were used. Three transplants from each experimental unit were sampled for growth analysis, $^{14}CO_2$ fixation assay and stomatal evaluation 2 and 4 weeks after transfer to soil and the final survival rates recorded.

Analysis of variance was performed using the General Linear Model procedure (GLM) of the Statistical Analysis System (SAS) (SAS Inc., 1985) on the means of each experimental unit. Homogeneity of variance was tested using the Bartlett's test (Steel and Torrie, 1980) and appropriate transformations were used where necessary (Gomez and Gomez, 1984).

5.4 Results

At the end of the 4 week treatment period 99% of red raspberry shoots survived regardless of treatment. The percentage of rooted red raspberry shoots (plantlets) under CO_2 enrichment was significantly higher (71%) as was their vigor rating (3.7) compared with those under ambient CO_2 (37% and 2.5, respectively). The amount of rooting and the general vigor of plantlets were not affected by the in vitro RH. The percentage rooting and the vigor rating of plantlets grown in media with 0, 1, and 5 $g \cdot liter^{-1}$ sucrose were 52.9%, 45% and 64% and 2.8, 2.9 and 3.6, respectively. Plantlet height, fresh weight, and leaf area increased with medium sucrose level under both CO_2 and RH treatments (Fig. 5.2). Plantlets grown under enriched CO_2 were taller, heavier and had more leaf area compared with plantlets grown under ambient CO_2. Plantlets grown at ambient RH were higher, heavier and had more leaf area than those grown under reduced RH. The number of leaves and roots and total root lengths were not affected by medium sucrose levels. Plantlets grown under enriched CO_2 had more (2.1) and longer roots (6.3 cm) compared with those grown under ambient CO_2 (1.7 and 7.5 cm, respectively). The number of leaves per plantlet were not affected by in vitro CO_2, RH or medium sucrose levels.

Two weeks after transfer to soil, there was no apparent residual effect of medium sucrose level on transplant growth.

Fig. 5.2. Effects of medium sucrose on stem heights, fresh weights and leaf areas of red raspberry plantlets under ambient and enriched CO_2 or ambient and reduced RH conditions. Values are the means of 9 samples.

Equations for CO_2:

Stem height:

Enriched CO_2 o $Y=10.42+0.40X$ $R^2=0.63$

Ambient CO_2 • $Y=8.73+0.51X$ $R^2=0.91$

Fresh weight:

Enriched CO_2 o $Y=220.84+22.47X$ $R^2=0.93$

Ambient CO_2 • $Y=192.74+19.45X$ $R^2=0.98$

Leaf area:

Enriched CO_2 o $Y=617.75+110.94X-17.44X^2$ $R^2=0.99$

Ambient CO_2 • $Y=312.25+342.81X-49.81X^2$ $R^2=0.99$

Equations for RH:

Stem height:

Ambient RH o $Y=9.21+0.43X$ $R^2=0.96$

Reduced RH • $Y=10.4+0.58X$ $R^2=0.89$

Fresh weight:

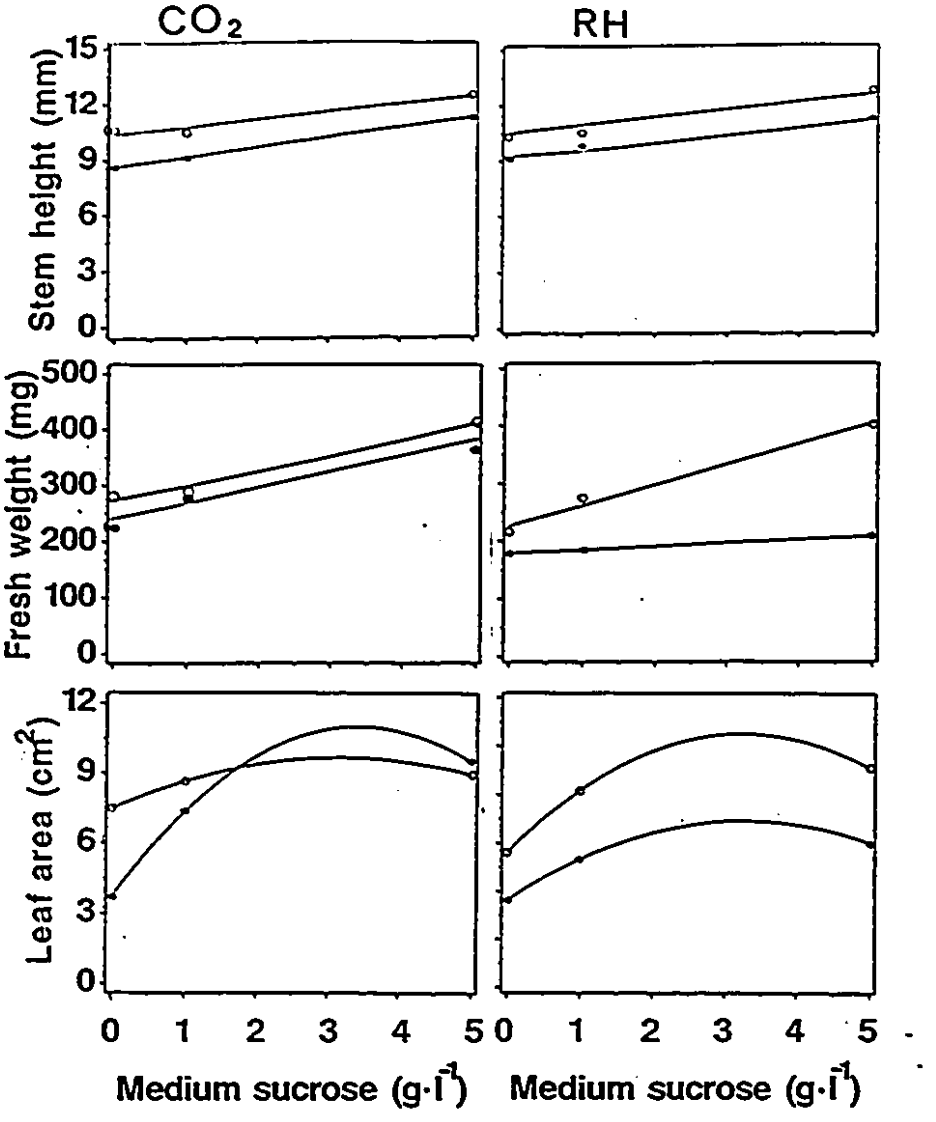
Ambient RH o $Y=179.56+5.89X$ $R^2=0.88$

Reduced RH • $Y=227.09+35.41X$ $R^2=0.98$

Leaf area:

Ambient RH o $Y=362.78+202.13X-30.71X^2$ $R^2=0.99$

Reduced RH • $Y=561.50+303.40X-46.65X^2$ $R^2=0.99$


CO2
RH
Stem height (mm)
15
12
9
6
3
0
Fresh weight (mg)
500
400
300
200
100
0
Leaf area (cm2)
12
9
6
3
0
0 1 2 3 4 5
0 1 2 3 4 5
Medium sucrose (g·l-1)
Medium sucrose (g·l-1)

However, the effects of in vitro CO_2 and RH become more obvious (Fig. 5.3). The stem heights, shoot fresh weights, and leaf areas were greater in transplants originally grown under in vitro reduced RH or enriched CO_2 compared with ambient RH or CO_2 (Fig. 5.3).

Four weeks after transfer to soil the residual effects on transplants of in vitro CO_2 and RH were still apparent. Shoot fresh weights and leaf areas of transplants originally grown under in vitro reduced RH or enriched CO_2 were greater than those under ambient RH or CO_2 (Fig. 5.3). All other parameters examined (stem heights, number of persistent leaves and new leaves and root fresh weights) were not related to in vitro CO_2, RH or medium sucrose levels. The final survival rates 4 weeks after transfer to soil were better for transplants originally grown under in vitro reduced RH (95%) or enriched CO_2 (99%) compared with ambient RH (85%) or CO_2 (81%).

The $^{14}CO_2$ activity levels in cultured plantlet leaves averaged 69% of control plants rates (Table 1). In vitro $^{14}CO_2$ activity levels were negatively related to the medium sucrose concentrations under all CO_2 and RH conditions (Fig. 5.4). The $^{14}CO_2$ activity levels were higher in 3 out of 4 leaves examined on plantlets grown under enriched compared with ambient CO_2 conditions (Fig. 5.5). In vitro RH level did not affect the $^{14}CO_2$ uptake ability of cultured plantlet leaves. Two and 4 weeks after transfer to soil, the $^{14}CO_2$

Fig. 5.3. Effects of in vitro CO_2 and RH conditions on the stem heights, fresh weights and leaf areas of red raspberry transplants 2 and 4 weeks after transfer to soil. Values are the means of 36 samples. Means within CO_2 or RH treatments were separated by LSD at 5% level.

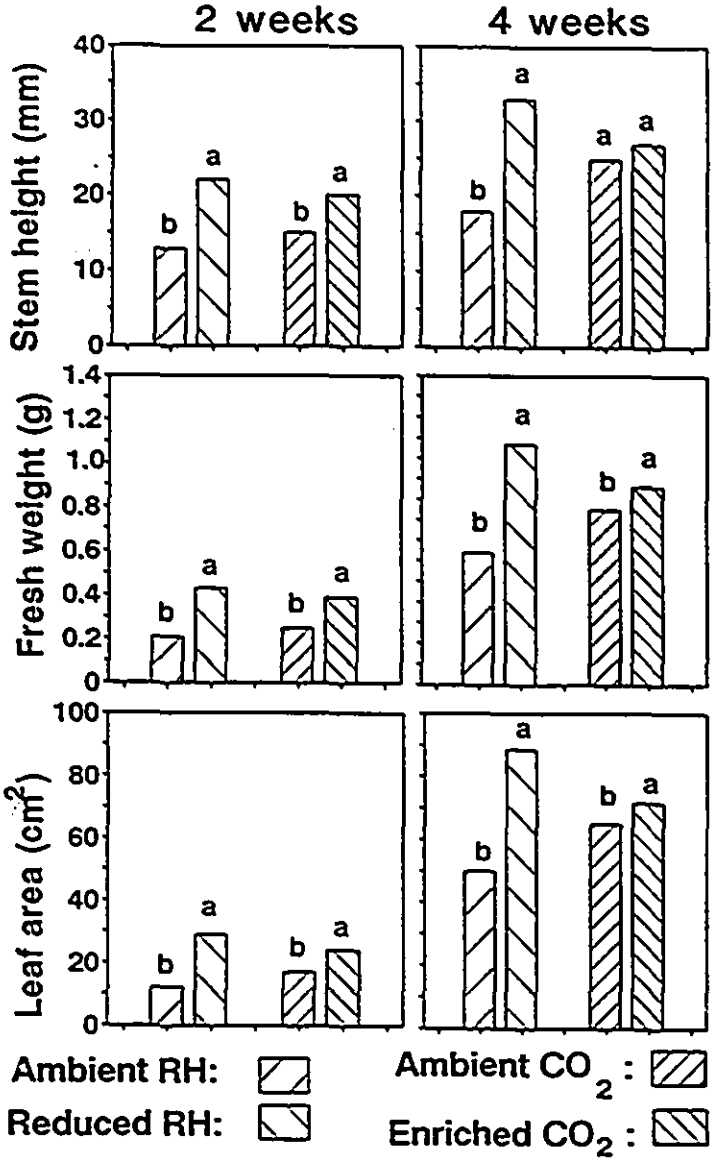

2 weeks
4 weeks
Stem height (mm)
Fresh weight (g)
Leaf area (cm²)
Ambient RH:
Reduced RH:
Ambient CO2 :
Enriched CO2 :

Table 5.1. The ^{14}C activity levels in cultured plantlet leaves, persistent and new leaves of red raspberry transplants and greenhouse control plant leaves after exposure to 740 KBq $^{14}CO_2$ for 5 min. Values represent the mean of 72 leaves and their activity as a percentage of the control plant leaves (mean of 5 leaves for control plant leaves).

Ex vitro time(wk)	Leaves	Activity (Kdpm•cm^{-2})	Percentage of Control (%)
0	plantlet	61.7 ± 3.8	69.0
2	persistent	54.2 ± 3.6	60.6
	new 1	48.4 ± 2.3	54.1
	new 2	56.3 ± 3.2	63.0
	persistent	49.7 ± 3.7	55.6
	new 1	50.6 ± 3.3	56.6
4	new 2	58.7 ± 4.0	65.6
	new 3	76.9 ± 6.0	86.0
	new 4	82.6 ± 6.7	92.4
	Control	89.4 ± 4.4	100

Fig. 5.4. The ^{14}C activity levels ($Kdpm \cdot cm^{-2}$) in leaves of cultured red raspberry plantlets as affected by medium sucrose levesl. Leaves were numbered basipetally. Values are the means of 9 samples.

Leaf No. 1 • $Y=78.55-12.67X+1.67X^2$ $R^2=0.98$

Leaf No. 2 ○ $Y=78.30-4.06X$ $R^2=0.97$

Leaf No. 3 △ $Y=61.80+3.07X-1.12X^2$ $R^2=0.95$

Leaf No. 4 ◇ $Y=54.32+0.35X$ $R^2=0.97$

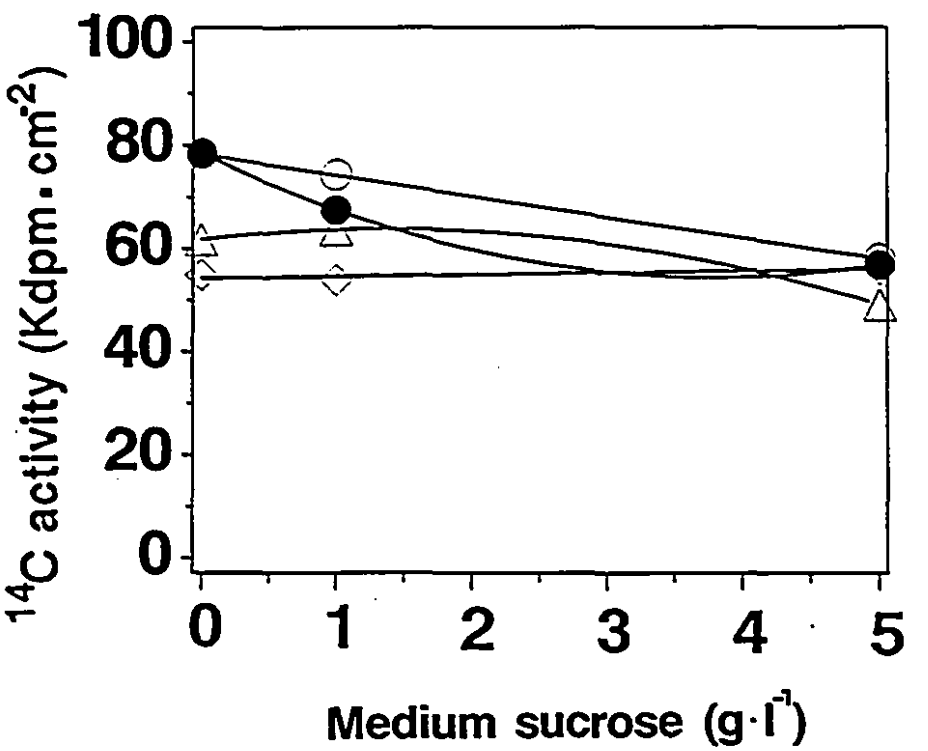
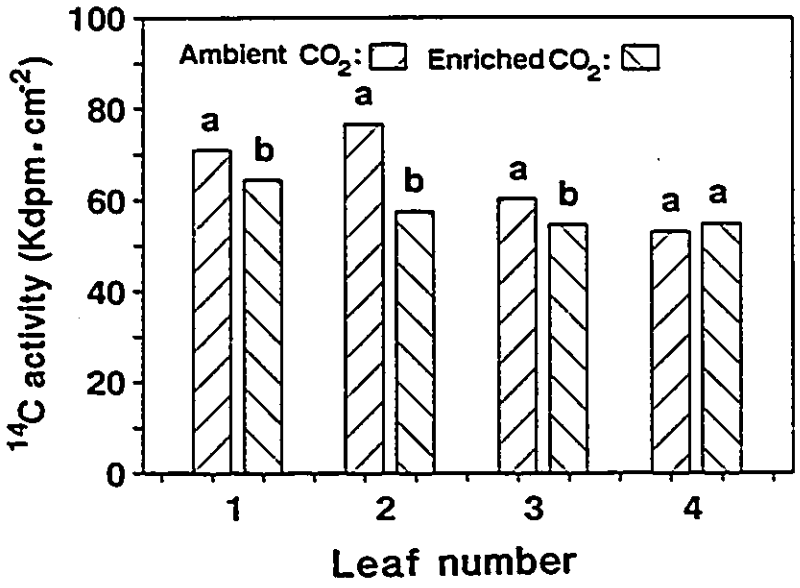

100
80
60
40
20
0
^{14}C activity (Kdpm·cm^{-2})
0
1
2
3
4
5
Medium sucrose (g·l^{-1})

Fig. 5.5. Effect of in vitro CO_2 conditions on the ^{14}C activity ($Kdpm \cdot cm^{-2}$) in leaves of cultured red raspberry plantlets. Leaves were numbered basipetally. Values are the means of 36 samples. Means within each leaf were separated by LSD at the 5% level.


Ambient CO_2:
Enriched CO_2:
^{14}C activity (Kdpm·cm^{-2})
100
80
60
40
20
0
a
b
a
b
a
b
a
a
1
2
3
4
Leaf number

uptake capacities of persistent leaves and new leaves were not related to in vitro CO_2, RH or medium sucrose treatment levels. The $^{14}CO_2$ uptake capacity of plantlet leaves was 69% that of control plant leaves and the $^{14}CO_2$ uptake capacity of persistent leaves of 4-week-old transplants was 56% that of control plant leaves. Consectively developed new leaves ex vitro had increasingly higher $^{14}CO_2$ uptake capacity with the fourth new leaf reaching 92.4% that of control plant leaves.

After 4 weeks in culture, S.I. of leaves of plantlets grown under enriched CO_2 were greater (0.14) compared with those grown under ambient CO_2 (0.12). Stomata were open in vitro and stomatal apertures were similar during the day and night in all treatments; stomata were not functional (Table 5.2). Stomatal apertures were larger on plantlet leaves grown under enriched (5.55 μm) compared with ambient (3.63 μm) CO_2 and ambient (5.25 μm) RH compared with reduced (3.90 μm) RH. Medium sucrose levels did not affect S.I. or aperture size of plantlet leaves.

Two weeks after transfer to soil, the stomatal apertures on persistent leaves were slightly smaller than those on cultured plantlet leaves. However, there was still no indication that the stomata closed at night (Table 5.2). Neither S.I., stomatal apertures nor percentage of open stomata on persistent leaves and the first new leaf were affected by in vitro CO_2, RH or medium sucrose level.

Four weeks after transfer to soil, the S.I., stomatal

Table 5.2. Stomatal characteristics of cultured plantlet leaves, persistent and mature new leaves of 2-week-old transplants. Values represent the mean of 72 leaves.

Leaves	Aperture (μM)		% open		Stomatal
	Day	Night	Day	Night	Index
Plantlet	4.6	4.4	89.0	91.3	0.13
Persistent	3.5	3.1	73.0	71.0	0.13
New 1	2.7	2.5	69.3	40.2	0.15
New 2	2.5	-	65.0	5.5	0.14
New 3	2.4	-	55.0	7.9	0.14

apertures and percentage of open stomata on the first three new leaves developed ex vitro were not affected by in vitro CO_2, RH or medium sucrose level. About 60% of the stomata on the first new leaves closed at night; clearly these stomata possessed the closure mechanism (Table 5.2). More than 90% of the stomata on the second and the third new leaves closed at night indicating an improvement in their functional status (Table 5.2). The stomatal apertures of these leaves during the day were similar to those of greenhouse control plant leaves (2.6 ± 0.3 μm) (Table 5.2)

5.5 Discussion

Under conventional micropropagation conditions, the CO_2 levels in culture vessels rapidly reached the CO_2 compensation point (≤ 100 ppm) during the light period and become excessively high (up to 2 - 5%) during the dark period (reviewed by Kozai, 1991). Limited CO_2 availability in culture vessels during the light period was one of the constraints to development of photosynthetic capacity of cultured plantlets. Excessively high CO_2 levels during the dark period might depress the growth of cultured plantlets (De Proft et al., 1985). In our study, gas exchange was greatly improved by forced ventilation to open vessels within plexiglass incubation chambers. The plantlets experienced the actual pre-set levels of CO_2 and some degree of air

turbulence. Enriched CO_2 treatment significantly promoted growth, photosynthetic rates and root formation of red raspberry plantlets (Fig. 5.2 and 5.4).

Previous CO_2 enrichment experiments involved either increasing the CO_2 levels in growth rooms (Kozai, 1991; Kozai et al., 1991a,b) or in specially constructed growth chambers (Desjardins et al., 1988; Laforge et al., 1991; Kozai & Iwanami, 1988; Kozai et al., 1990c) but the culture vessels remained closed. The growth of strawberry plantlets cultured in the photoautotrophic tissue cultured system (PTCS) which provided 300 - 330 ppm CO_2 levels, 96 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 93% RH were 71.9% higher compared with plantlets grown in their standard tissue culture system which provided 150 - 240 ppm CO_2 levels, 42 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and saturated RH (Fujiwara et al., 1988). The fresh weight of strawberry plantlets grown on sugar-free medium under enriched CO_2 (2,000 ppm in culture room during light period) was 85.9% and 59.0% heavier than those grown at ambient CO_2 on medium with 0 or 20 $g \cdot liter^{-1}$ sucrose (Kozai et al., 1991a). The dry weight of tobacco plantlets grown under enriched CO_2 (700 to 4,200 ppm in cultured room) was more than twice those grown under ambient CO_2 regardless of light intensity (from 61 to 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$), with or without sucrose after 30 days of incubation (Kozai et al., 1990c). Similarly, in this study, the growth (stem heights, fresh weights and leaf areas) of red raspberry plantlets grown under enriched CO_2 was consistently

greater compared with those grown under ambient CO_2 (Fig. 5.2) and their residual effect could carry over up to 4 weeks ex vitro (Fig. 5.3).

The net photosynthetic rates of strawberry plantlets cultured in the PTCS were 775% higher compared with plantlets grown in their standard tissue culture system (Fujiwara et al., 1988). The net photosynthetic rates of cultured *Actinidia deliciosa* L. were 4 times higher under 1,450 or 4,500 ppm CO_2 compared with ambient CO_2 (Infante et al., 1989). Similarly, in our study, the CO_2 uptake rates of red raspberry plantlets were higher under enriched than ambient CO_2 conditions (Fig. 5.5). Low CO_2 levels were indeed limiting the CO_2 uptake rates of cultured red raspberry plantlets and it was possible to improve the CO_2 uptake rates by increasing the CO_2 levels.

In vitro CO_2 enrichment had a positive effect on plantlet growth and CO_2 uptake but increased leaf S.I. and stomatal apertures. The latter would tend to promote stomatal evapotranspiration. However, ex vitro performance was not affected by this, probably as a consequence of improved root development (more and longer roots).

Reduced trichome numbers, decreased foliar waxes and non-functional stomata with wide apertures on cultured plantlet leaves were related to the saturated (100% RH) in vitro atmosphere (Sutter, 1982; Sutter and Langhans, 1979). These features lead to excessive water loss by evapotranspiration

which contributes to the desiccation of conventional propagates. Reducing in vitro RH using desiccants (Short et al., 1987; Wardle et al., 1983; Ziv et al., 1983), increased agar concentration (Ziv et al., 1983), porous closures (Short et al., 1987; Smith et al., 1990) or bottom-cooling (Maene & Debergh, 1987; Capellades et al., 1990) caused leaves in vitro to more closely resemble the control phenotype. The 90% RH level used in this study reduced stomatal apertures greatly (3.90 μm) compared with stomatal apertures of plantlets grown at ambient RH (5.25 μm) but stomata were not all functional since they had similar apertures during the days and nights (Table 5.2). Although the plantlets grown under reduced RH had less fresh weight and shorter stems compared with those grown at ambient RH (Fig. 5.2), the former appeared healthier and their ex vitro performance was better (Fig. 5.3). In vitro RH of less than 80 - 85% had adverse effects on plantlet growth (Short et al., 1987; Ziv, 1986). However, less than maximal growth at reduced RH in vitro may be offset by improved growth ex vitro.

The $^{14}CO_2$ uptake capacity of photoautotrophic plantlets was greater than that of mixotrophic plantlets (Fig. 5.4). Although photoautotrophic plantlets were less vigorous compared with mixotrophic plantlets (Fig. 5.2), they performed similarly ex vitro. Medium sucrose did not affect the behavior of persistent leaves on 2 or 4 week old transplants. The $^{14}CO_2$ uptake rates of plantlet leaves were about 69% that

of control plants and dropped to 55.6% by 4 weeks ex vitro. Persistent leaves of red raspberry could last up to 3 months ex vitro under conventional acclimatization (Donnelly & Vidaver, 1984a,b; Donnelly et al., 1985). The CO_2 uptake ability of the persistent leaves were relatively high. They substantially contributed to transplant growth. Photoautotrophic transplants could adapt to ex vitro conditions more quickly than the mixotrophic transplants and thus had a shorter period of acclimatization.

The first few consecutively developed ex vitro new leaves were transitional; exhibiting gradually higher CO_2 uptake rates than cultured plantlet leaves and persistent leaves and the CO_2 uptake rates of the fourth ex vitro new leaves reached that of control leaves. In contrast, under conventional micropropagation and with tenting of stage IV plantlets there were a minimum of 5 weeks were required for acclimatization, leaves formed the fifth week resembles control leaves but had not achieved control levels of photosynthesis (Donnelly et al., 1984). The number of transitional leaves formed on transplants appear to depend on the degree of hardening of cultured plantlets and the stress imposed by the new ex vitro environment. The fewer the number of transitional leaves, the briefer the acclimatization interval. The reduced number of transitional leaves produced on transplants in the current study compared with earlier reports for micropropagated red raspberry suggested that plantlets cultured under forced

ventilation were hardened and close to control plants phenotypically.

Plantlets may be considered hardened if they can survive transplantation to ex vitro ambient conditions with minimal or no extra precautions, have relatively high photosynthetic capacity and narrow stomatal apertures.. Hardened plantlets may still share certain features of the CIP and will produce transitional leaves after transplantation. Transplants may be considered fully acclimatized when new leaves represent the control phenotype; have photosynthetic capacity comparable to that of control plants and functional stomata. In other words, transitional leaves are no longer formed, leaves with control type phenotype are produced.

In vitro hardened photoautotrophic red raspberry plantlets were established on sucrose-free medium using a system of forced ventilation. Their ex vitro performance was superior to that of mixotrophic plantlets. These in vitro hardened plantlets could be transferred directly to soil under ambient greenhouse conditions without any special precautions.

Preface to Chapter 6

Chapter 6 is a reproduction of a manuscript by R. Deng and D.J. Donnelly which was presented orally by R. Deng at the annual meeting of the Atlantic Canada Plant Tissue Culture Association held at Woodstock P.E.I. from July 4 to 5, 1991. This manuscript was accepted by the Canadian Journal of Plant Science in November 1992. This chapter gives a comprehensive discussion of the in vitro hardening of red raspberry through CO_2 enrichment and relative humidity reduction on sugar-free medium.

Chapter 6 In vitro hardening of red raspberry through CO_2 enrichment and relative humidity reduction on sugar-free medium

6.1 Abstract

Micropropagated shoots of red raspberry (*Rubus idaeus* L. 'Comet') were rooted on modified Murashige-Skoog medium lacking sucrose, in specially constructed plexiglass chambers, under ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2 and ambient (ca. 100%) or reduced (90 ± 5%) relative humidity. Cultured plantlets were evaluated for their survival, rooting and relative vigor, leaf and root numbers, stem and root lengths, total leaf areas, total fresh and dry weights, gas exchange rates, and stomatal features, prior to transplantation to soil and at intervals up to 6 weeks ex vitro. In vitro CO_2 enrichment promoted plantlet growth, rooting and both the survival and early growth of transplants. CO_2 enrichment increased stomatal apertures of plantlet leaves but did not apparently increase water stress at transplantation. Reduced in vitro RH did not affect plantlet growth but decreased stomatal apertures and stomatal index on leaves of cultured plantlets and promoted both the survival and early growth of transplants. In vitro CO_2 and RH levels did not affect the photosynthetic rates of either plantlets or transplants. Only the stomata on leaves of plantlets from the

ambient CO_2 and reduced RH treatment were functional. Normal stomatal function was not observed in persistent leaves of transplants from the other treatments, even 2 weeks ex vitro. In vitro CO_2 enrichment acted synergistically with RH reduction in improving growth of plantlets both in vitro and ex vitro. Hardened red raspberry plantlets obtained through CO_2 enrichment and RH reduction survived direct transfer to ambient greenhouse conditions without the necessity for specialized ex vitro acclimatization treatment.

6.2 Introduction

In vitro-grown plants possess a unique culture-induced phenotype (CIP) (Donnelly et al., 1985) which includes anatomical and physiological features different from those of greenhouse- or field-grown plants. The CIP is characterized by tiny shoots and plantlets with decreased epicuticular and cuticular wax, reduced mechanical support tissue, increased percentage water content, non-functional stomata and mixotrophy (reviewed by Donnelly and Tisdall, 1992). The CIP explains ex vitro transplant stress and mortality which result from poorly-controlled water loss and reduced photosynthetic competence. Traditionally, an acclimatization period of several weeks follows ex vitro transplantation of micropropagates. During this time the transplants are weaned from high humidity and the light is slowly increased to

ambient levels. In ex vitro *Rubus*, field- or greenhouse-grown type anatomy and physiology gradually developed through a series of transitional organs (Donnelly and Vidaver, 1984b; Donnelly et al., 1984). Ex vitro acclimatization is expensive and time consuming; contributing significantly to the overall cost and the difficulty of commercial micropropagation (Kozai, 1991; Vasil, 1991). Alternatively, changes in the culture environment, especially in the later stages of micropropagation, can promote hardening-off in vitro. Modifying the CIP towards improved photosynthetic competence or improved water relations reduces the length of or eliminates the need for ex vitro acclimatization.

Sucrose in the medium stimulates the growth of cultured plantlets but at the commonly used rate (3%) or higher it depresses photosynthesis and results in mixotrophy. Rose (*Rosa multiflora* L.) plantlets cultured on medium with 1% sucrose had 10 times the photosynthetic rates of those on 5% sucrose (Capellades et al., 1991). Cauliflower (*Brassica oleraceae* var. Botrytis) plantlets cultured on sugar-free medium had photosynthetic rates 115% greater than those grown on medium with 3% sucrose (Short et al., 1987). Autotrophy was promoted in several other species when the sugar in the medium was reduced or eliminated (reviewed by Kozai, 1991).

Autotrophy was also promoted when the carbon dioxide concentration and, in some cases, the light levels were increased (reviewed by Kozai, 1991). The growth and net

photosynthetic rates of strawberry plantlets cultured in the "photoautotrophic tissue cultured system" which provided 300 - 330 ppm CO_2 levels, 96 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 93% RH were 72% and 775% higher, respectively, compared with plantlets grown under standard tissue culture conditions which provided 150 - 240 ppm CO_2 levels, 42 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and saturated RH (Fujiwara et al., 1988). The fresh weight of strawberry plantlets grown on sugar-free medium under enriched CO_2 (2,000 ppm in the culture room during the light period) was 86% and 59% heavier, respectively, than those grown at ambient CO_2 on media with 0 or 20 $g \cdot liter^{-1}$ sucrose (Kozai et al. 1991). The photosynthetic rates of cultured *Actinidia deliciosa* plantlets were 4 times higher under 1,450 or 4,500 ppm CO_2 compared with those grown under ambient CO_2 (Infante et al., 1989). The dry weight of tobacco plantlets grown under enriched CO_2 (700 to 4,200 ppm in the culture room) was more than twice that of plantlets grown under ambient CO_2 regardless of light intensity (from 61 to 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and with (20 $g \cdot liter^{-1}$) or without sucrose after 30 days of incubation (Kozai et al., 1990c).

Wax deposition on leaves of cultured chrysanthemum (*Chrysanthemum morifolium* L.) (Sutter, 1982) and carnation (Sutter and Langhans, 1979) plantlets was negligible at saturated (100%) in vitro RH. There was a negative relationship between in vitro RH and the amount of foliar wax on leaves of cultured cauliflower (Short et al., 1987).

Leaves had the least (20 $mg \cdot cm^{-2}$) foliar wax when grown under saturated RH (100%) compared with those grown under 80% RH (49 $mg \cdot cm^{-2}$). Well developed foliar epicuticular wax was observed on cauliflower plantlets cultured under reduced in vitro RH (30%) brought about by covering the media with lanolin (Wardle et al., 1983). 'Moutse' rose plantlets cultured under 75% RH and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity had foliar wax and stomata similar to leaves of greenhouse-grown plants (Capellades et al., 1990). Stomata on leaves of grape plantlets cultured at 94% RH closed, while those cultured at 100% RH did not, following exposure to 20% RH for 18 h in darkness (Smith et al., 1992).

While the anatomy and physiology of cultured plants were improved by reduced in vitro RH, growth and multiplication rates declined, especially when the RH was below 80% (Ziv et al., 1983; Ziv, 1986). Short et al. (1987) found that the growth of cultured cauliflower and chrysanthemum plantlets was severely hindered when the RH was below 70%. In preliminary experiments with red raspberry, RH levels below 90% severely inhibited plantlet growth and caused rapid medium desiccation.

No investigation has yet been carried out to determine the effects of in vitro CO_2 enrichment and RH reduction on plantlet growth and photosynthetic capacity in vitro and subsequently on ex vitro transplants. In this study, plexiglass chambers were utilized to create an aseptic environment in which CO_2 could be supplemented and RH lowered

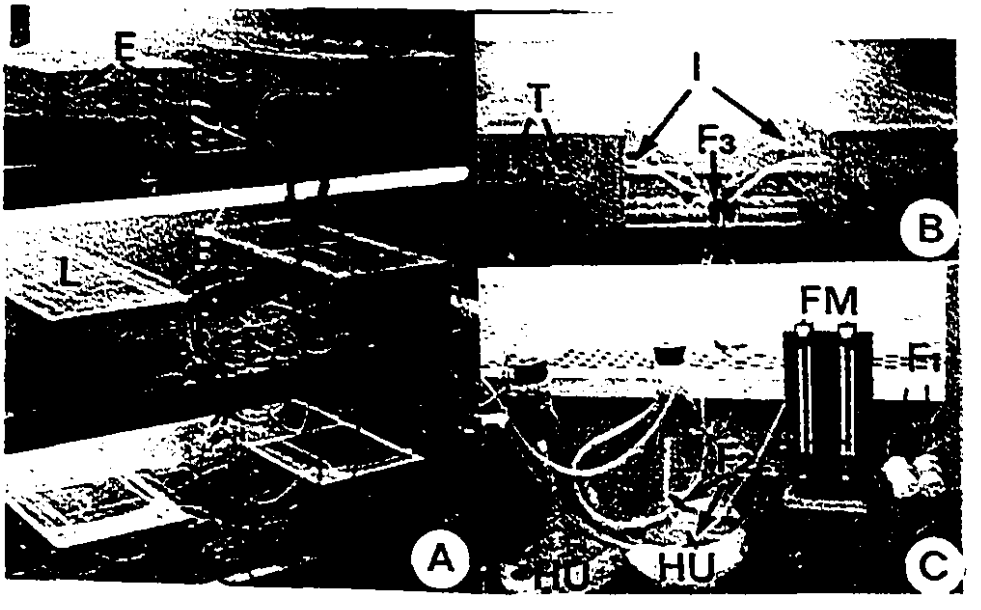
to study in vitro hardening of red raspberry (*Rubus idaeus* L.) plantlets. The objective was to produce hardened plantlets for which conventional ex vitro acclimatization procedures of tenting, misting or fogging would not be necessary.

6.3 Materials and methods

Specially designed plexiglass chambers (55 x 30 x 15 cm) with tightly-fitting plexiglass lids, secured with elastic bands wrapped around paired hooks, were used to house open culture vessels exposed to controlled atmospheres (Fig. 6.1). The streams of CO_2 gas mixtures from compressed cylinders were regulated by a flow meter and scrubbed with a series of filters before they were humidified in a 4.5 L erlenmeyer and buffered in small erlenmeyer flasks that preceded the incubation chambers. Each chamber had two air inlets and two air outlets on the opposite side and three sensor probe ports; two for temperature and one for humidity. The chambers and tubing systems were surface sterilized with 10% bleach and 70% ethanol, respectively.

'Comet' red raspberry was micropropagated as previously described (Donnelly and Vidaver 1984a). Shoots with 2 to 3 leaves and fresh weights of 23 ± 4 mg were rooted on Murashige and Skoog (1962) basal salt medium without sucrose supplemented with 1.2 μM thiamine·HCl, 550 μM inositol, 2.45 μM indole-3-butanoic acid (IBA) and 5.5 g•liter^{-1} agar

Fig. 6.1. The experimental set up showing the CO_2 and RH control systems and the plexiglass incubation chambers. Tight fitting plexiglass lids were secured to the chambers (C) with elastic bands (E) wrapped around paired hooks. CO_2 gas mixtures traveled from compressed gas cylinders through a flow meter (FM) and a series of filters (F1 = in line, type 407, ADC; F2 = syringe type, 0.4 μm and F3 = syringe type, 0.2 μm) and were humidified in a large erlenmeyer (HU) before entering a buffering chamber (B) which preceded the incubation chambers (C). Air entered each incubation chamber through two air inlets (I) and flushed out through two air outlets on the opposite side. The temperature and RH in the chambers were monitored through the two temperature probe ports (T) and one RH probe port (H)


E
L
A
T
I
F3
B
FM
F1
HU
C

(Anachemia) adjusted to pH 5.7.

The treatments included 4 weeks of incubation under ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO_2 and ambient (ca. 100%) or reduced (90 ± 5%) RH. The four factorial treatment combinations were arranged in the plexiglass chambers according to a randomized complete block design with four replicates. Each treatment replicate consisted of 4 uncovered, 500 ml plastic culture vessels, each containing 70 ml of rooting medium and 9 shoots. Pre-mixed and analyzed gas mixtures of either ambient or enriched CO_2 were continuously supplied to the chambers from compressed cylinders at a flow rate of 15 $ml \cdot min^{-1}$. Chamber RH was maintained at either 100 or 90% by bubbling the gas mixtures through 2 L of either pure water or 45% glycerol (v/v) (modified from Forney and Brandl, 1992) in 4.5 L erlenmeyer flasks.

The cultures were maintained at 25 ± 3C with a light intensity at plant height of 55 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (cool white fluorescent) and a 16 h photoperiod. Attempts to increase the light intensity further were frustrated by excessive diurnal temperature variations within the chambers. The RH and temperature inside the chambers were monitored at intervals through probe-ports in the plexiglass chambers with a Humicor, IHRT digital RH/Temperature indicator and a telethermocouple (Cole-Parmer, Model 8500-40, Chicago, Il).

After 4 weeks, survival, rooting and relative plantlet

vigor (scale of 1 to 5) were evaluated. For each treatment, 4 randomly selected plantlet leaves were detached for determination of water loss over a period of 180 min (Crane and Hughes 1990) and 4 plantlets were used for gas exchange measurements then for counts of leaf and root numbers and measurements of stem and root lengths, total leaf areas and total fresh and dry weights.

Twenty-four plantlets from each treatment, each with its youngest leaf tagged with colored thread, were transferred to cell packs containing 2:1 Promix (BX):loam soil in a growth chamber at 30 - 40% RH, 25 ± 3C under cool white fluorescent lights of 120 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400-700 nm) with a 16 h photoperiod. Five transplants from each treatment were harvested for counts of numbers of persistent and new leaves and measurements of stem length, fresh weight of shoots and roots and total leaf areas at 2 and 4 weeks ex vitro. The final survival rates were recorded at 6 weeks ex vitro.

Photosynthetic rates of intact plantlets, 4-week-old ex vitro transplants before and after removal of their persistent leaves (Donnelly and Vidaver, 1984b) and detached leaves of greenhouse-grown control plants were measured with an infrared gas analyzer (IRGA) (ADC 225). Measurements were made under ambient CO_2 conditions (ca. 340 ± 20 ppm) at 25 ± 3C in closed-system mode (Long and Hallgren, 1985). The light intensity at leaf surface level was 100 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400-700 nm) provided by 3 cool-white fluorescent tubes.

At each sampling time for gas exchange measurements four leaves were also randomly sampled for chlorophyll a, b, a+b and total carotenoid pigment contents (Sestak et al., 1971). One, 2 and 6 weeks ex vitro, 8 persistent leaves, and, when available, 8 first new leaves (the first new leaf above the tagged leaf) and 8 second new leaves (the second new leaf above the tagged leaf) were randomly selected from each treatment for measurements of stomatal index and aperture. Four leaves were taken 5 hours after the start of the light period and another 4 leaves taken 5 hours after the start of the dark period and immediately fixed and cleared for light microscopy (Tisdall and Donnelly, 1988). Five random, abaxial fields of view from each leaf, each with about 60 epidermal cells and about 10 stomata, were photographed and the detailed measurements made on the prints.

Analysis of variance was performed on the data using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS Inc., 1985). Homogeneity of variance was tested using Bartlett's test (Steel and Torrie, 1980) and transformations were used where necessary (Gomez and Gomez, 1984).

6.4 Results

All shoots and plantlets grew on medium without sugar under ambient or enriched CO_2 and ambient or reduced RH.

Rooting was superior (73.2%) and plantlets more vigorous (4.3) under enriched compared with ambient CO_2 (39.8%, 2.5). In vitro RH of either 100% or 90% did not affect rooting or plantlet vigor (data not shown). Detached leaves of plantlets from all treatments lost water more rapidly than greenhouse control plant leaves (Fig. 6.2). Almost all of the water lost from plantlet leaves (60-80%) occurred during the first 60 min. Detached leaves from plantlets grown under ambient CO_2 and reduced RH lost 18% less water than leaves from the other treatments, which all lost similar amounts of water.

The carbon dioxide fixation rates at the time of transplantation were similar (2.8 ± 1.1 mg CO_2 $dm^{-2} \bullet h^{-1}$) for leaves of plantlets from all treatments. Four weeks after transplantation the average photosynthetic capability of persistent leaves had decreased (1.7 ± 0.5 mg CO_2 $dm^{-2} \bullet h^{-1}$) but that of the new leaves was considerably greater (6.2 ± 1.8 mg CO_2 $dm^{-2} \bullet h^{-1}$). Greenhouse control plant leaves had CO_2 fixation rates of 15.0 ± 1.2 mg CO_2 $dm^{-2} \bullet h^{-1}$.

In vitro CO_2 enrichment increased root (72.5%) but not leaf numbers, stem (28%) and root (100%) lengths, total leaf areas (69%) and fresh (70.5%) and dry (63.5%) weights of plantlets compared with those grown under ambient CO_2 conditions (Table 6.1). In vitro RH of 100% or 90% did not affect any of these growth parameters (data not shown). Two weeks ex vitro, the numbers of persistent and new leaves were unaffected but shoots and roots of transplants from reduced in

Fig. 6.2. In vitro CO_2 (340 ± 20 ppm, 1500 ± 50 ppm) and RH (100%, 90%) conditions affected the amount of water lost from detached red raspberry plantlet leaves. Each data point represents the mean of 16 leaves. Bars indicate the 95% confidence intervals for average water lost from all leaves for each of 4 blocks.

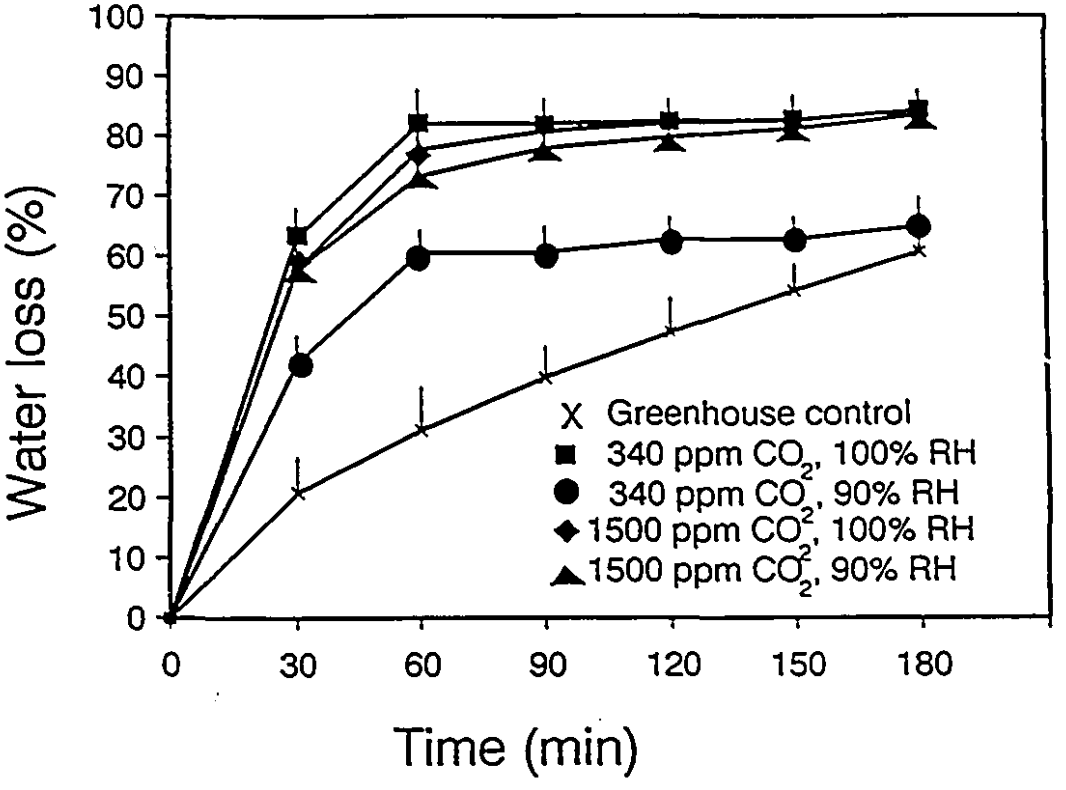

Water loss (%)
100
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
Time (min)
X Greenhouse control
340 ppm CO2, 100% RH
340 ppm CO2, 90% RH
1500 ppm CO2, 100% RH
1500 ppm CO2, 90% RH

Table 6.1. The influence of in vitro CO_2 levels on various growth parameters of red raspberry plantlets.

CO_2	Number		Length (cm)		Leaf area	Weight (mg)	
(ppm)	leaves	roots	stems	roots	(cm_2)	fresh	dry
340	9.0	4.0	0.57	38.2	46.6	220.6	28.2
1500	10.1	6.9	0.73	76.0	78.9	376.1	46.1
Sig.[a]	ns	*	*	**	**	**	**

[a] *, ** and ns significant at P=<0.05, or 0.01 and non-significant, respectively.

vitro RH and enriched CO_2 were 33% and 16% heavier, respectively, and had 35% more leaf area than those from other in vitro treatments (Table 6.2). The residual effect of CO_2 enrichment in culture carried over 2 weeks ex vitro as indicated by heavier transplant shoots and roots and much greater total leaf area compared with transplants from ambient CO_2 conditions in vitro (Table 6.2). Two-week-old transplants from in vitro enriched CO_2 and reduced RH had the highest shoot and root fresh weights and leaf areas followed by those from enriched CO_2 and ambient RH, then by those from ambient CO_2 and both ambient and reduced RH (Table 6.2). However, after 4 weeks ex vitro treatment effects were no longer apparent. All transplants had similar numbers of persistent (2.4) and new leaves (5.4), stem lengths (2.7 cm), fresh weights of shoots (1245 mg) and roots (159 mg), and total leaf areas (585.5 cm^2).

Transplant survival after 6 weeks ex vitro was 97% from the in vitro-enriched CO_2 treatments but only 70% from the ambient CO_2 treatments. Transplant survival was 87% from the in vitro reduced RH treatments and 79% from the ambient RH treatments.

Chlorophyll a and total carotenoid contents were greater in plantlet leaves under enriched than ambient CO_2 conditions (2.29 and 0.60 compared with 1.60 and 0.10 $mg \cdot g^{-1}$ fresh weight, respectively). Chlorophyll b contents were not affected by CO_2 levels and averaged 1.05 $mg \cdot g^{-1}$ fresh weight.

Table 6.2. The influence of in vitro CO_2 and RH conditions on the subsequent ex vitro growth of red raspberry transplants 2 weeks after transfer to soil.

CO_2 (ppm)	RH (%)	Leaf number		Fresh weight (mg)		Leaf area (cm^2)
		pers.*	new	shoots	roots	
340	100	6.3 a	2.3 a	400 c	85.5 c	91.2 c
340	90	6.8 a	2.4 a	350 c	79.4 c	64.0 c
1500	100	7.2 a	2.8 a	640 b	116.4 b	136.3 b
1500	90	7.6 a	2.8 a	850 a	134.7 a	183.3 a

* pers = persistent.

Within columns, means followed by the same letter are not significantly different by DMRT at the 5% level.

In vitro RH levels did not affect foliar pigment contents (data not shown). Chlorophyll a (1.89 $mg \cdot g^{-1}$ fresh weight), b (0.88 $mg \cdot g^{-1}$ fresh weight) and total carotenoid (0.79 $mg \cdot g^{-1}$ fresh weight) contents were all similar among leaves of 4-week-old transplants regardless of treatments.

Stomatal index (SI) of leaves of plantlets grown under ambient CO_2 and reduced RH (13.2 ± 0.3) was less than in other treatments (14.5 ± 0.4) as was aperture size (Table 6.3). Plantlets from the ambient CO_2 and reduced RH treatment had stomata that were apparently functional, opening during the day and closing at night. Plantlets from the other treatments all had nonfunctional stomata since their apertures did not vary diurnally (Table 6.3). Stomata of persistent leaves of 1-week-old transplants were not functional since their apertures did not vary diurnally (Table 6.3). Persistent leaves of 1-week-old transplants had smaller stomatal apertures than leaves of plantlets in vitro. Plantlets grown under ambient CO_2 and reduced RH had persistent leaves with permanently closed stomata unlike the persistent leaves from plants of other treatments where stomata were nonfunctional and remained permanently open. Stomatal characteristics were similar on persistent leaves 1 and 2 weeks ex vitro (data not shown). SI and aperture sizes of the first and second new leaves of 2- and 6-week-old transplants were not affected by in vitro CO_2 or RH conditions. The stomatal apertures on the first and second ex vitro new leaves of 2- and 6-week-old

Table 6.3. The influence of in vitro CO_2 and RH conditions on stomatal apertures (μm) on detached plantlet leaves and persistent leaves of 1-week-old ex vitro transplants evaluated during the day and night.

CO_2 (ppm)	RH (%)	Plantlet leaves		Persistent leaves	
		Day	Night	Day	Night
340	100	5.29 b	5.50 a	5.00 b	4.84 a
340	90	2.82 d	1.65 c	2.09 d	2.17 c
1500	100	6.28 a	6.01 a	5.76 a	5.18 a
1500	90	3.71 c	4.51 b	3.26 c	2.92 b

Within columns, means followed by the same letter are not significantly different ($p=<0.05$).

transplants were 3.15 ± 0.25 and 2.75 ± 0.21 μm during the day and 2.63 ± 0.20 and 2.43 ± 0.25 μm during the night, respectively. The stomatal apertures on greenhouse-grown leaves were 2.60 ± 0.3 μm during the day and 2.10 ± 0.20 μm during the night, respectively.

6.5 Discussion

Leaves of micropropagated red raspberry plantlets were photosynthetically competent. The persistent leaves of ex vitro transplants continued to contribute photosynthetically for 1 month. However, CO_2 uptake decreased as these leaves senesced; by almost 61% after 4 weeks ex vitro.

Plantlet growth (Table 6.1), but not photosynthetic capacity, was increased under enriched CO_2 conditions in vitro. Increased growth resulting from CO_2 enrichment has been observed in a wide range of species (reviewed by Kozai, 1991). Elevated CO_2 levels promote stomatal opening (Morison, 1987) and may explain the increased aperture size of foliar stomata in the CO_2 enrichment treatments. This apparently contributed to poor water retention ability of detached leaves (Fig. 6.2), but did not increase water stress at transplantation probably due to the improved root development of plantlets in these treatments. The residual effects of CO_2 supplementation in vitro carried over for only 2 weeks (Table 6.2), unlike the 4 week carry-over noted with strawberry by

Laforge et al. (1991). The promotive effects of elevated in vitro CO_2 were further increased when the RH was reduced to 90% (Table 6.2). All surviving transplants were similar in size by 4 weeks ex vitro indicating the lack of residual effects from in vitro CO_2 and RH treatments. In vitro CO_2 enrichment increased both chlorophyll a and total carotenoids in leaves of plantlets to levels comparable with leaves of transplants or field control plants. This might be essential for the plantlets to adapt to higher light intensity ex vitro. All new transplant leaves, regardless of in vitro treatments, had similar pigment contents suggesting that they were no longer influenced by in vitro treatments.

'Comet' plantlet growth and photosynthesis were not greatly affected by reducing in vitro RH to 90%. The photosynthetic CO_2 uptake of rose plantlets grown at 75% and 85% RH was 65% and 91% that of control plantlets grown at 100% RH (Capellades, 1989). In vitro RH reduction caused reduced water loss from detached leaves (Fig. 6.2) and improved ex vitro transplant performance (Table 6.2).

Transplants from the reduced RH and ambient CO_2 conditions experienced less water stress as a result of functional stomata (Table 6.3) and lower SI. RH reduction in vitro to 90% was apparently necessary to the normal function of stomata (Table 6.3) and did not greatly compromise raspberry plantlet growth. However, stomatal function did not occur in plantlets grown under ambient CO_2 and RH or in the

enriched CO_2 treatments. Nonfunctional stomata were also observed on leaves of conventionally micropropagated apple (*Malus pumila* L.) (Brainerd and Fuchigami, 1982), cherry (*Prunus cerasus* L.) (Marin et al., 1988) and carnation (Ziv, et al., 1987). Our enriched CO_2 level of 1500 ppm may have been too high for normal functional development of stomata. Stomatal conductance of sunflower (*Helianthus annuus* L.) decreased by 90% when the CO_2 concentration was increased from 340 ppm to 1,000 ppm indicating the closure of stomata. However, the stomata reopened when the CO_2 levels were increased above 1,000 ppm (Robinson et al., 1988).

Stomatal function had not improved in persistent 'Comet' leaves by 2 weeks ex vitro, in contrast to reported improvements seen in cherry (Marin et al., 1988) and apple (Brainerd and Fuchigami, 1982). Stomatal apertures on persistent leaves of 'Comet' transplants were narrower than on plantlet leaves in vitro (Table 6.3) which might partly reflect the improved water relations of older transplants. The stomata on the first ex vitro developed new leaves closed slightly at night indicating a weak function. Stomata on the second ex vitro developed new leaves (these appeared at the end of the first week ex vitro) had stomata resembling those of control leaves. New leaves formed the fifth week ex vitro from conventionally micropropagated red raspberry transplants resembled greenhouse-grown control plant leaves anatomically (Donnelly and Vidaver, 1984a, b; Donnelly et al., 1984).

Culture closures are necessary in conventional micropropagation systems to prevent contamination. Closures impede air exchange between container contents and the outside atmosphere and may encourage the accumulation of hazardous gases such as ethylene (De Proft et al., 1985). In the open culture system used in this study plantlets were exposed to CO_2 levels of at least 340 ppm at all times, unlike cultures in conventional micropropagation systems that experience diurnal CO_2 levels close to the compensation point (<100 ppm) during the light periods and excessively high CO_2 levels (up to 9000 ppm or more) during the dark periods (reviewed by Kozai, 1991). Plantlets grown at ambient CO_2 levels had CO_2 fixation rates similar to those grown under enriched CO_2 but the higher CO_2 level promoted plantlet and subsequent transplant growth (Table 6.1, 6.2). The CO_2 uptake rates of plantlets and transplants in this study were similar to conventionally micropropagated raspberry in sucrose-containing medium in a closed system (Donnelly and Vidaver, 1984b). This suggests that other factors, such as light, were limiting. Increased in vitro light intensity, up to several times higher than normal, promoted the growth and photosynthetic competence of a wide range of species (reviewed by Kozai, 1991). Our attempts to increase the light intensity beyond 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ were frustrated by diurnal temperature variations within the chambers. However, this may be overcome by using chambers equipped with temperature regulators (Laforge et al., 1990).

This is among the first reports of successful in vitro hardening resulting in direct transfer of plantlets to ambient greenhouse conditions without the need for ex vitro acclimatization treatment of any kind. RH reduction acted synergistically with CO_2 enrichment in improving the growth and some aspects of the anatomy and physiology of plantlets. Further improvements may be achieved by optimizing the in vitro CO_2 and RH levels and other aspects of the culture environment including light intensity. In vitro hardening of micropropagated red raspberry has significant commercial implications.

Chapter 7 General conclusion

Field performance of micropropagated (MP) and conventionally propagated (CP) red raspberry (*Rubus idaeus* L. cv. Comet and Festival) was examined under hedgerow and stool management systems for 3 seasons (1989 to 1991) at Macdonald Campus. MP plants, produced in our laboratory or purchased from a commercial micropropagator, were superior to CP bare-rooted canes obtained from a certified Quebec propagator; their establishment was clearly better and they maintained an advantage for three growing seasons. Micropropagated red raspberry planting stock clearly showed promise for both nursery propagation and fruit production.

MP plants were less vulnerable than CP plants to field losses as indicated by 100% survival among the MP plant population compared with 58% survival among the CP population after field transplantation followed by 92% survival after replanting. MP plants established better and were more vigorous than CP plants during this study as indicated by greater cane height, more and larger primocanes per plant in 1989 and more primocanes per plant in 1990 and 1991. The up to four-fold increases in number of primocanes per plant in the MP compared with the CP plants would greatly benefit the nursery industry.

The propagation methods did not affect the photosynthetic capacity of red raspberry primocane and floricane leaves under

field conditions. The general pattern of photosynthetic activity of primocane leaves during the growing season was typical of field crops. They usually started out with lower photosynthetic rates early in the season and then increased in early summer before declining in the middle of summer and increasing again later in the growing season. The photosynthetic capacity of floricane leaves showed a distinct pattern related to the fruit development. The photosynthetic rates of floricane leaves were relatively high at the beginning of anthesis (28 May to 2 June) then decreased toward the end of anthesis (15-20 June) but increased corresponding to the period of rapid berry development (20-30 June) and decreased again toward the beginning of berry ripening (early July).

Winter hardiness of both cultivars was not affected by propagation method or cane management system. Leaf and stem morphology were consistent within each cultivar regardless of plant source.

MP 'Festival' plants produced a crop equivalent to 2.2 $MT \cdot ha^{-1}$ while yields from CP 'Festival' and MP and CP 'Comet' were negligible the first fruiting year. The MP 'Festival' crop (8.42 $MT \cdot ha^{-1}$) also outyielded CP 'Festival' (6.8 $MT \cdot ha^{-1}$) and both MP (5.72 $MT \cdot ha^{-1}$) and CP (4.91 $MT \cdot ha^{-1}$) 'Comet' in the second fruiting year. The increased fruit yields in MP 'Festival' plants might be derived partly from greater numbers of flowers and fruit on longer laterals. However, yields of

'Comet' were unaffected by propagation method.

Cane management systems had no effect on field performance of MP or CP plants in this relatively young plantation due to insufficient cane numbers to fully implement the management systems.

Red raspberry plantlets were successfully hardened in vitro on low-sucrose or sucrose-free media through CO_2 enrichment (1500 ppm) and RH reduction (90%) using a forced ventilation system in specially constructed plexiglass chambers. Gas exchange between the interior and the exterior of culture vessels was improved to open culture vessels by forced ventilation to the incubation chambers. The plantlets experienced the actual set levels of CO_2 as well as some degree of air turbulence. These in vitro hardened plantlets could be transferred directly to soil under ambient greenhouse conditions without any special precautions. Enriched CO_2 significantly increased general vigor, root formation, root growth (increased root number and root length), plantlet growth (increased plantlet fresh and dry weight) and plantlet photosynthetic capacity. The benefit of CO_2 enrichment was especially significant when the plants were cultured on sucrose-free media. CO_2 enrichment increased leaf stomatal index and stomatal apertures but did not cause more water stress ex vitro possibly due to improved root characterestics. Root hairs were abundant and long on the root tips of photoautotrophic plantlets compared with the very short root

hairs on the root tips of mixotrophic plantlets. The enormous number of longer root hairs on the root tips of photoautotrophic plantlets likely decreased water stress after transplantation.

This research confirmed that in vitro CO_2 levels were limiting photosynthetic rates of cultured red raspberry plantlets and that by increasing the CO_2 levels in vitro it was possible to improve their CO_2 uptake rates with associated improvement in in vitro hardening. The residual effects of enriched CO_2 on plantlet growth persisted up to 2 to 4 weeks after transplantation ex vitro.

Sucrose in the medium promoted plantlet growth but depressed photosynthesis under ambient CO_2 conditions, however, the benefit of sucrose in the media was limited under enriched CO_2 conditions. Photoautotrophic plantlets were obtained on sucrose-free rooting medium under forced ventilation and they performed better during ex vitro acclimatization compared with mixotrophic plantlets grown on sucrose-containing media.

The maximum CO_2 uptake rates of plantlet leaves were about 52 - 69% that of greenhouse control plant leaves and did not change in the persistent leaves up to 4 weeks after ex vitro transplantation. These results indicated that the CO_2 uptake abilities of cultured and persistent leaves were relatively high and substantially contributed to transplant growth. The photosynthetic ability of persistent and new

leaves of 4-week-old ex vitro transplants related neither to in vitro CO_2 exposure levels nor to the medium sucrose concentrations.

Consecutively developed ex vitro new leaves had gradually higher CO_2 uptake rates than cultured plantlet leaves and persistent leaves; they were transitional leaves. The CO_2 uptake rates of the fourth ex vitro new leaves reached that of control leaves which suggested that the first three ex vitro new leaves were transitional leaves.

In vitro relative humidity at 90% decreased stomatal apertures and improved plantlet ex vitro performance, but did not affect the CO_2 uptake rates of cultured plantlets or ex vitro transplants. However, all stomata on cultured plantlet leaves were not functional and remained so up to 4 weeks after ex vitro transplantation. Normal functional stomata occurred on the second ex vitro new leaves. Stomatal apertures on persistent leaves of 2- or 4-week-old transplants were narrower than on plantlet leaves which might partly reflect the improved water relations of transplants.

Plantlets may be considered hardened if they can survive ambient conditions with minimal or no extra precautions when transferred ex vitro, have relatively high photosynthetic capacity and narrow stomatal aperture with or without functional ability. Hardened plantlets may still share certain features of the CIP and produce transitional leaves after transplantation to ambient greenhouse conditions.

Transplants may be considered fully acclimatized when new leaves represent the control phenotype with photosynthetic capacity comparable to that of control plants and functional stomata. Once fully acclimatized transplants can be moved successufully to field conditions.

The number of transitional leaves formed on transplants appears to depend on the degree of hardening of the cultured plantlets and the stress imposed by the new ex vitro environment. The fewer the number of transitional leaves, the briefer the acclimatization interval. The reduced number of transitional leaves produced on transplants in the current study compared with earlier reports for micropropagated red raspberry suggested that plantlets cultured under forced ventilation were hardened and closer to control plants phenotypically.

This is among the first reports of successful in vitro hardening resulting in direct transfer of plantlets to ambient greenhouse conditions without the need for special treatment of any kind. In vitro hardening of micropropagated red raspberry has significant commercial implications.

Chapter 8 Contribution to original knowledge

1. This is the first report of a long-term field evaluation of micropropagated red raspberry. This study provided valuable field performance data demonstrating that MP red raspberry, produced in our laboratory or purchased from a commercial micropropagator, was superior to CP bare-rooted canes, from a certified propagator, as planting material for both nursery propagation and fruit production. This study showed that MP red raspberry established better than CP bare-rooted canes under Quebec field conditions; MP red raspberry were more vigorous than CP plants throughout the duration of this study (3 years) as indicated by greater cane height, increased number of leaves per cane and more and larger primocanes per plant in 1989 and more primocanes per plant in 1990 and 1991; MP red raspberry produced up to four times the number of primocanes per plant per year and the quality (diameter and height) of primocanes was the same as those produced by CP plants. This information will have significance implications for the commercial red raspberry propagation industry.

2. This study confirmed that the photosynthetic capacity of red raspberry primocane and floricane leaves under field conditions was not affected by propagation. This is the first report of the photosynthetic activity pattern of red raspberry

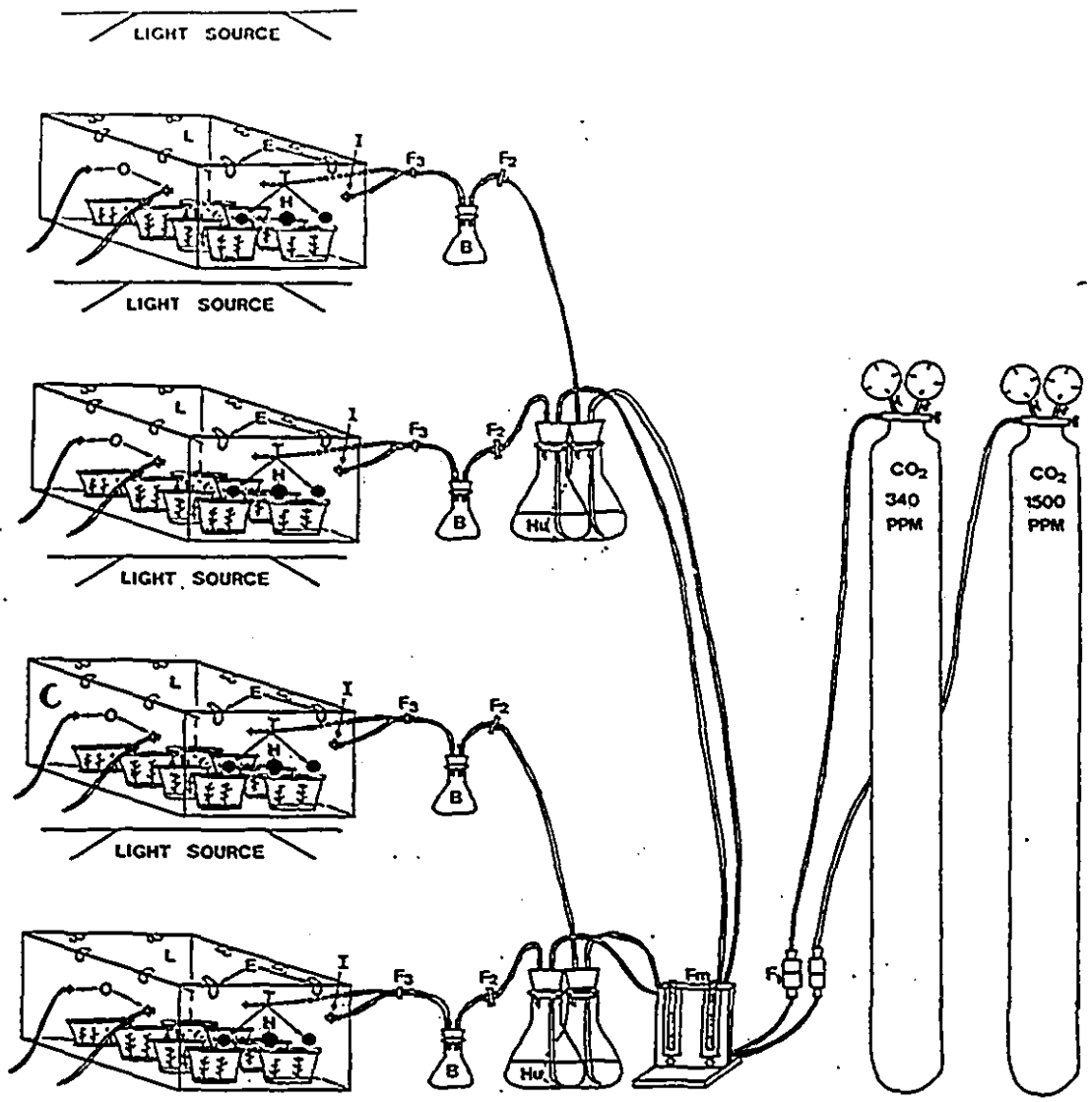
floricane leaves in relation to fruit development.

3. This study showed that the leaf and stem morphology was consistent within each cultivar and was not affected by micropropagation. This is the first report that winter hardiness of 'Comet' and 'Festival' red raspberry was not affected by propagation method. This finding will pave the way for the more wide-spread commercial use of MP red raspberry since risk of off-type variation is of little concern.

4. MP 'Festival' plants produced a crop equivalent to 2.2 $MT \cdot ha^{-1}$, i.e. commercially harvestable, the second year after planting (the first fruiting year). The commercial implication of this finding is that by utilizing MP planting stock there is the potential to advance commercial fruit production for some cultivars by 1 year. This study showed that the red raspberry fruit yield of MP 'Festival' was significantly higher than that of CP 'Festival' during the first and the second fruiting year while the fruit yield of 'Comet' red raspberry was not affected by micropropagation. The berry size was not affected by micropropagation.

5. This is the first report of an original system using open vessels held within sterile incubation chambers to root red raspberry under forced ventilation (Fig. 8.1). The CO_2 and RH

Fig. 8.1 The experimental set up showing the CO_2 and RH control systems and the plexiglass incubation chambers. Tight fitting plexiglass lids were secured to the chambers (C) with elastic bands (E) wrapped around paired hooks. CO_2 gas mixtures traveled from compressed gas cylinders through a flow meter (FM) and a series of filters (F1 = in line, type 407, ADC; F2 = syringe type, 0.4 μm and F3 = syringe type, 0.2 μm) and were humidified in a large erlenmeyer (HU) before entering a buffering chamber (B) which preceded the incubation chambers (C). Air entered each incubation chamber through two air inlets (I) and flushed out through two air outlets on the opposite side. The temperature and RH in the chambers were monitored through the two temperature probe ports (T) and one RH probe port (H)


LIGHT SOURCE
L
E
I
T
H
F3
F2
B
LIGHT SOURCE
Hu
LIGHT SOURCE
C
LIGHT SOURCE
Fm
F1
CO2
340
PPM
CO2
1500
PPM

levels inside the chambers were precisely controlled simultaneously. Gas exchange was thus improved to open vessels by forced ventilation. The plantlets experience the actual set levels of CO_2 and RH as well as some degree of air turbulence in the immediate vicinity. Under the light regime utilized (55 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$), the temperature fluctuation within the chambers remained within 2C of the room temperature. However, for this system to be utilized with higher light intensities a heat exchang device would be ecessary. This is a relatively inexpensive system, it shows great promise not only as a research tool but could also be readily scale up for commercial micropropagation.

6. This is the first report demonstrating that red raspberry shoots were successfully rooted and hardened in vitro on low-sucrose or sucrose-free media through CO_2 enrichment and RH reduction using a forced ventilation system in specially constructed plexiglass chambers. These in vitro hardened plantlets could be transferred directly to soil under ambient greenhouse conditions without any special precautions. Thus the conventional acclimatization period was eliminated, a significant potential saving for commercial micropropagators.

7. This study confirmed that low CO_2 levels in vitro were indeed limiting the CO_2 uptake of cultured red raspberry plantlets. Enriching CO_2 levels in vitro to 1500 ppm

significantly increased general vigor, root formation, root growth (increased root number and length), plantlet growth (increased plantlet fresh and dry weight) and photosynthetic capacity. However, CO_2 enrichment increased leaf stomatal index and stomatal aperture which tend to encourage evapotranspiration.

8. This is the first report of long abundant root hairs on root tips of red raspberry plantlets grown on sucrose-free medium. The enormous number of long root hairs on the root tips of these plantlets would tend to improve root function after transplantation. Root tips from plantlets grown on medium containing any amount of sucrose had short root hairs.

9. This is the first report of a relationship between stomatal aperture of red raspberry and in vitro CO_2 level i.e. CO_2 at 1500 ppm increased stomatal aperture to 5.55 μm compared with 3.63 μm at ambient CO_2. This is also the first report of a relationship between stomatal aperture of red raspberry and in vitro RH level, i.e. RH at 90% decreased stomatal aperture to 3.90 μm compared with 5.25 μm at 100% RH. This study showed that stomata on cultured plantlet leaves were not functional and remained of 2 - 4 weeks after ex vitro transplantation. However, the stomatal apertures of persistent leaves of transplants were narrower than those of plantlet leaves.

10. This is among the first attempts to define hardened plantlets and acclimatized transplants. Plantlets were considered hardened if they could survive ex vitro ambient conditions with minimal or no extra precautions after transplantation, had relatively high photosynthetic capacity and narrow stomatal apertures with or without functional ability. Hardened plantlets may still share certain features of the CIP and produce transitional leaves after transplantation. Transplants were considered fully acclimatized when transitional leaf morphology were no longer formed and new leaves represented the control phenotype; with photosynthetic capacity comparable to that of control plants and functional stomata.

11. This study showed that the maximum CO_2 uptake rates of hardened red raspberry plantlet leaves were about 52 - 69% that of greenhouse control plant leaves and did not change in the persistent leaves up to 4 weeks after transplantation. These results indicated that the CO_2 uptake abilities of hardened plantlets and the persistent leaves of transplants were relatively high and contributed substantially to transplant growth.

12. This study confirmed that during ex vitro acclimatization consecutively developed new leaves had gradually higher CO_2 uptake rates than cultured plantlet leaves and persistent

leaves; they were transitional leaves. The CO_2 uptake rates of the third or the fourth ex vitro developed new leaves reached that of control leaves. At this point the transplants were said to be fully acclimatized to ex vitro condition.

Chapter 10 Literature Cited

Agriculture Canada. 1984. Cultivating red raspberries in eastern Canada. Publication 1762.

Anderson, W.C. 1980. Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *R..occidentalis*. Acta Hort. 112:13-20.

Anonymous, 1992. Statistiques Agricoles. Statistiques De L' Agriculture des Peches et de L'Alimentation. Edition 1992.

Babic, V., and M. Neskovic. 1984. Propagation of three blackberry cultivars from small apical bud in vitro. J. Hort. Sci. 59:183-185.

Bakker, J.C. 1991. Effects of humidity on stomatal density and its relation to leaf conductance. Sci. Hort. 48:205-212.

Bailey, L.H. 1941. Species Bartorum. the genus Rubus in North America I. Gentes Herb. 5:1-64.

Bailey, J.S. 1949. Winter injury to red raspberries as affected by cultivation or mulching. Proc. Amer. Soc. Hort. Sci. 54:197-199.

Barlass, M., and K.G.M. Skene. 1980. Studies on the fragmented shoot apex of grapevine, II: factors affecting growth and differentiation in vitro. J. Expt. Bot. 31:489-495.

Bedard, R., and A. Garneau. 1985. Field evaluation of tissue-cultured strawberry plants. HortScience 20:522(abstr.).

Blanke, M.M., and A.R. Belcher. 1989. Stomata of apple leaves cultured in vitro. Plant Cell, Tissue and Organ Culture.

19:85-89.

Brainerd, K.E., and L.H. Fuchigami. 1981. Acclimatization of aseptically cultured apple plant to low relative humidity. J. Amer. Soc. Hort. Sci. 106, 515-518.

Brainerd, K.E., and L.H. Fuchigami. 1982. Stomatal functioning of in vitro and greenhouse apple leaves in darkness, mannitol, ABA and CO_2. J. of Exp. Bot. 33: 388-392.

Brainerd, K.E., L.H. Fuchigami, S. Kwiatkowski, and C.S. Clarke. 1981. Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments. HortScience 16:173-175.

Braun, J.W., and J.K.L. Garth. 1984. Intracane yield compensation in the red raspberry. J. Amer. Soc. Hort. Sci. 109:526-530.

Braun, J.W., J.K.L. Garth., and C.A. Brun. 1989. Distribution of foliage and fruit in association with light microclimate in the red raspberry canopy. J. Hort. Sci. 64:565-572.

British Columbia Ministry of Agriculture and Fisheries. 1987. British Columbia Raspberry Certification Program and guidelines. pp. 8.

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

Buonassisi, A.J., H.A. Daubeny, and B. Peters. 1989. The B.C. raspberry certification program. Acta Hort. 262:175-180.

Buszard, D.J. 1986. The effect of management system on winter survival and yield of raspberries in Quebec. Acta Hort.

183:175-181.

Cameron, J.S., and J.F. Hancock. 1986. Enhanced vigor in vegetative progeny of micropropagated strawberry plants. HortScience 21:1225-1226.

Cameron, J.S., J.F. Hancock, and J.A. Flore. 1989. The influence of micropropagation on yield·components, dry matter partitioning and gas exchange characteristics of strawberry. Sci. Hort. 38: 61-67.

Cameron, J.S., J.F. Hancock, and T.M. Nourse. 1985. The field performance of strawberry nursery stock produced originally from runners or micropropagation. Adv. Strawberry Prod.4:56-58.

Cancellier, S., and F. Cossio. 1988. Field observations on a clone of 'Corvina veronese (*Vitis vinifera* L.) multiplied by 'in vitro' culture. Acta Hort. 508-513.

Capellades, M. 1989. Histological and ecophysiological study of the changes occurring during the acclimatization of in vitro cultured roses. Ph.D. Diss., State Univ., Gent, Belgium.

Capellades, M., R. Fontarnau, C. Carulla, and P. Debergh. 1990. Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa multiflora*. J. Amer. Soc. Hort. Sci. 115:141-145.

Capellades, M., R. Lemeur, and P. Debergh. 1991. Effects of sucrose on starch accumulation and rate of photosynthesis in Rosa cultured in vitro. Plant Cell, Tissue and Organ Culture

25:21-26.

Chamber, J. 1961. The production and maintenance of virus-free raspberry plants. J. Hort. Sci. 36:48-54.

Chu, I.Y.E., and S.L. Kurtz. 1990. Commercialization of plant micropropagation. In: Sharp, W.R., Evans, D.A., Ammirato, P.V, and Yamada, Y (eds.). Hand book of plant cell culture. Vol. 5. Macmillan Publishing Company. pp. 126-164.

Conner, L.N., and A.J. Conner. 1984. Comparative water loss from leaves of *Solsanum laciniatum* plants cultured in vitro and in vivo. Plant Sci. Let. 36:241-246.

Conner, A.J., and Thomas, M.B., 1981. Re-establishing plantlets from tissue culture: a review. Comb. Proc. Int. Plant Prop. Soc. 31:342-357.

Cormack, M.R., H.M. Lawson, and P.D. Waister. 1976. Comparisons of four methods of establishing raspberry plantations from spawn canes. Hort. Res. 16:1-17.

Craig, D.L., and L.E. Aalders. 1966. Winter injury and yielding ability of six red raspberry varieties under two systems of culture in Nova Scotia. Can. J. Plant Sci. 46:73-76.

Crandall, P.C., J.D. Chamberlain, and K.A. Biderbost. 1974a. Cane characteristics associated with berry number of red raspberry. J. Amer. Soc. Hort. Sci. 99:370-372.

Crandall, P.C., D.F. Allmendinger, J.D. Chamberlain, and K.A. Biderbost. 1974b. Influence of cane number, diameter, irrigation and carbohydrate reserve on the fruit number of

red raspberries. J. Amer. Soc. Hort. Sci. 99:524-526.

Crandall, P.C., J.D. Chamberlain, and J.K.L. Garth. 1980. The effects of chemical primocane suppression on growth, yield, and chemical composition of red raspberries. J. Amer. Soc. Hort. Sci. 105:194-196.

Crandall, P.C., and H.A. Daubeny. 1990. Raspberry management. In: Small fruit crop management. Galletta, G.J. and D.G. Himelrick, (eds.). Prentice Hall. U.S.A. pp. 157-213.

Crane, J., and H. Hughes. 1990. Medium overlays for improved hardening of micropropagated potatoes. HortScience 25:794-795.

Dale, A. 1987. Field propagation of red raspberry canes. HortScience 22:950.

Dale, A. 1989. Productivity in red raspberry. Hort. Rev. 11:185-228.

Dale, A., and H.A. Daubeny. 1985. Genotype-environment interactions involving British and Pacific Northwest red raspberry cultivars. HortScience 20:68-69.

Dale, A., and A. Vandenberg. 1989. Guideline of Ontario raspberry plant propagation program. Horticulture Research Institute of Ontario (H.R.I.O). Canada.

Dalman, P. 1989. Within-plant competition and carbohydrate economy in the red raspberry. Acta Hort. 262:269-276.

Damiano, C. 1980. Strawberry micropropagation. In: Proc. conf. on nursery production of fruit plants through tissue culture-applications and feasibility. USDA-SEA, Agr. Res.

Results, ARR-NE-11. pp. 11-22.

Damiano, C., W. Faedi, and D. Cobianchi. 1983. Nursery runner production, fruiting and behavior of micropropagated strawberry plants. Acta Hort. 131:193-200.

De Proft, M.P., L.J. Maene, and P.C. Debergh. 1985. Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured in vitro. Physiol. Plant. 65:375-379.

Debergh, P.C. 1988. Micropropagation of woody species - state of the art on in vitro aspects. Acta Hort. 227:287-295.

Debergh, P.C. 1991. Acclimatization techniques of plants from in vitro. Acta Hort. 289:291-300.

Deng, R., and D.J. Donnelly. 1992a. A simple, cold extraction method for liquid sinctillation counting of ^{14}C-labeled red raspberry leaf tissue. Submitted to Can. J. Plant Sci.

Deng, R., and D.J. Donnelly. 1992b. In vitro hardening of red raspberry through CO_2 enrichment and sucrose reduction. Submitted to HortScience.

Desjardins, Y. 1990. Etude du comportement de vitroplants de fraisier (*Fragaria X ananassa* Duch.) et d'asperge (*Asparagus officinalis* L.) soumis a de hautes teneurs de I'atmosphere en CO_2 et a de hauts flux photoniques photosyntetiques en culture in vitro et en acclimatation. Ph.D. Diss. Laval Univ. Quebec, Canada.

Desjardins, Y, A. Gosselin, and S. Yelle. 1987. Acclimatization of in vitro strawberry plantlets under CO_2 enriched environment and supplemental lighting. J. Amer.

Soc. Hort. Sci. 112:S46-S51.

Desjardins, Y, F. Laforge, C. Lussier, and A. Gosselin. 1988. Effect of CO_2 enrichment and high photosynthetic photon flux on the development of autotrophy and growth of tissue-cultured strawberry, raspberry and asparagus plants. Acta Hort. 230:45-53.

Dhawan, V., and S.S. Bhojwani. 1987. Hardening in vitro and morpho-physiological changes in leaves during acclimatization of micropropagated plants of *Leucaenaleu cocephala* (Lam.) De Wit. Plant Sci. 53:65-72.

Donnan, A, Jr. 1986. Determining and minimizing production costs. In: Tissue culture as a plant production system for horticultural crop. Zimmerman, R.H. (Ed.). Martinus Nijhhoff Publisher pp. 167-173.

Donnelly, D.J., and H.A. Daubeny. 1986. Tissue culture of *Rubus* species (a review). Acta Hort. 183:305-314.

Donnelly, D.J. and L. Tisdall. 1992. Acclimatization strategies for micropropagated plants. In: Micropropagation of woody plants. Martinus Nijhoff Dr. W. Junk of Kluwer, Academic Publishers. Dr. W.R. Ahuja (Ed.). in press.

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

Donnelly, D.J., and W.E. Vidaver. 1984b. Pigment content and gas exchange of red raspberry in vitro and ex vitro. J. Amer. Soc. Hort. Sci. 109:177-181.

Donnelly, D.J., and Vidaver. W.E. 1987. Hydrathode structure of micropropagated plants and greenhouse-grown 'Totem' strawberry plants. J. Amer. Soc. Hort. Sci. 112:755-759.

Donnelly, D.J., F.E. Skelton, and H. A. Daubeny. 1986. External leaf features of tissue cultured 'Silvan' blackberry. HortScience 21:306-308.

Donnelly, D.J., F.E. Skelton, and J.E. Nelles. 1987. Hydathode anatomy and adaxial water loss in micropropagated 'Silvan' blackberry. J. Amer. Soc. Hort. Sci. 112:566-569.

Donnelly, D.J., R. Stace-Smith, and F.C. Mellor. 1980. In vitro culture of three Rubus species. Acta Hort. 112:69-73.

Donnelly, D.J., W.E. Vidaver, and K. Colbow. 1984. Fixation of $^{14}CO_2$ in tissue cultured red raspberry prior to and after transfer to soil. Plant Cell, Tissue and Organ Culture 3:313-317.

Donnelly, D.J., W.E. Vidaver, and K.Y. Lee. 1985. The anatomy of tissue cultured red raspberry prior to and after transfer to soil. Plant Cell, Tissue and Organ Culture 4:43-50.

Ellis, M.A., R.H. Converse, R.N. Williams, and B. Williamson. 1991. Compendium of raspberry and blackberry diseases and insects. APS Press pp. 100.

Evans, D.A., and J.E. Bravo. 1986. Phenotypic and genotypic stability of tissue cultured plants. In: Tissue culture as a plant production system for horticulture crops. Zimmerman, R.H. (eds.). Martinus Nijhoff Publishers, The Netherlands. pp. 73-94.

Fabbri, A., E. Sutter, and S.K. Dunston. 1986. Anatomical changes in persistent leaves of tissue culture strawberry plants after removed from culture. Sci. Hort. 28:331-337.

FAO. 1975. World production year book. Vol. 29.

FAO. 1989. World production year book. Vol. 43.

Fari, M., A. Andrasfalvy, and J. Nemeth. 1987. Thin PVC foil covering (TPFC), an efficient method for culture and preacclimatization of in vitro plant cultures. Acta Hort. 212:371-374.

Fernandez, G.E., and J.R. Clark. 1991. In vitro propagation of the erect thornless 'Navaho' blackberry. HortScience 26:1219.

Figueira, A., A. Whipkey, and J. Janick. 1991. Increased CO_2 and light promote in vitro shoot growth and development of *Theobroma cacao*. J. Amer. Soc. Hort. Sci. 116:585-589.

Forney, C.F., and D.G. Brandl. 1992. Control of humidity in small controlled-environment chambers using glycerol-water solutions. HortTechnology 2:52-54.

Freeman, J.A., and H.A. Daubeny. 1986. Effect of chemical removal of primocanes on several raspberry cultivars. Acta Hort. 183:215-222.

Freeman, J.A., G.W. Eaton, T.E. Baumann, H.A. Daubeny, and A. Dale. 1989. Primocane removal enhances yield components of raspberries. J. Amer. Soc. Hort Sci. 114:6-9.

Fuchigami, L.H., T.Y. Cheng, and A. Soeldner. 1981. Abaxial transpiration and water loss in aseptically cultured plum.

J. Amer. Soc. Hort. Sci.106: 519-22.

Fujiwara, K., T. Kozai, and I. Watanabe. 1987. Fundamental studies on environments in plant tissue culture vessels. (3) measurements of carbon dioxide gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets. J. Agric. Meteorol. 43:21-30 (Japanese with English summary).

Fujiwara, K., T. Kozai, and I. Watanabe. 1988. Development of a photoautotrophic tissue culture system for shoots and/or plantlets at rooting and acclimatization stages. Acta Hort. 230:153-158.

George, E.F., and P.D. Sherrington. 1984. Plant Propagation by tissue culture. Exegetic Limited. 709 pp.

Goff, L.M. 1986. Certification of horticultural crops -- a state perspective. In: Tissue culture as a plant production system for horticultural crop Zimmerman, R.H.(Ed.). Martinus Nijhhoff Publisher pp. 139-146.

Gomez, K.A., and A.A. Gomez. 1984. Statistical procedures for agricultural research. 2nd ed. John Wiley & Sons Inc. pp. 680.

Grout, B.W.W. 1975. Wax development on leaf surfaces of *Brassica oleracea* var. Currawong regenerated from meristem culture. Plant Sci. Let. 5:401-405.

Grout, B.W.W. 1988. Photosynthesis of regenerated plantlets in vitro, and the stresses of transplanting. Acta Hort. 230:129-135.

Grout, B.W.W., M.J. Aston. 1977. Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem generation. Hort. Res. 17:1-7.

Grout, B.W.W., and M.J. Aston. 1978a. Transplanting of cauliflower plants regenerated from meristem culture. II. carbon dioxide fixation and the development of photosynthetic ability. Hort. Res. 17:65-71.

Grout, B.W.W., and M.J. Aston. 1978b. Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. Ann. Bot. 42:993-995.

Grout, B.W.W., and P.C. Crisp. 1977. Practical aspects of the propagation of cauliflower by meristem culture. Acta Hort. 78:289-295.

Grout, B.W.W., and M.E. Donkin. 1987. Photosynthetic activity of cauliflower meristem cultures in vitro and at transplanting into soil. Acta Hort. 212: 323-327

Grout, B.W.W., and S. Millam. 1985. Photosynthetic development of micropropagated strawberry plantlets following transplanting. Ann. Bot. 55:129-131.

Grout, B.W.W., and F. Price. 1987. The establishment of photosynthetic independence in strawberry cultures prior to transplanting. In: G. Ducate, M. Jacobs and A. Simeon (eds.). Plant micropropagation in horticultural industries. Proc. Symp. Florizel, Arlon. pp. 55-60.

Grout, J.M., P.E. Read, and D.K. Wildung. 1986. Influence of

tissue culture and leaf bud propagation on the growth habit of 'Northblue' blueberry. J. Amer. Soc. Hort. Sci. 111:372-375.

Hammerschlag, F.A, and R. Scorza. 1991. Field performance of micropropagated, owned-rooted peach trees. J. Amer. Soc. Hort. Sci. 116:1089-1091.

Hancock, J.F. 1991. Photosynthesis in strawberries and the possibility of genetic improvement. In: The strawberries into the 21st century. Proc. 3rd N. Amer. Strawberry Conf. Dale, A. and J.J. Luby (Eds.). Timber Press, pp. 133-137.

Harper, P.C. 1978. Tissue culture propagation of blackberry and tayberry. Hort. Res. 18:141-143.

Hoepfner, A.S. 1989. In vitro propagation of red raspberry. Acta Hort. 262:285-288.

Honjo, T., Y. Futaya, and T. Takakura. 1988. Effect of CO_2 enrichment on the growth of *Cymbidium* PLB in vitro. Acta Hort. 230:185-188.

Howard, B.H., E. Tal, and S.K. Mitra. 1987. Red raspberry propagation from leafy summer cuttings. J. Hort. Sci. 62:485-492.

Howard, B.H., O.P. Jones, and J. Vasek. 1989a. Long-term improvement on the rooting of plum cuttings following apparent rejuvenation. J. Hort. Sci. 64:147-156.

Howard, B.H., O.P. Jones, and J. Vasek. 1989b. Growth characteristics of apparently rejuvenated plum shoots. J. Hort. Sci. 64:157-162.

Hudson, J.P. 1954. Propagation of plants by root cuttings. I. Regeneration of raspberry root cuttings. J. Hort. Sci. 29:27-43.

Hudson, J.P. 1959. Effects of environment on *Rubus idaeus* L. I. Morphology and development of the raspberry plants. J. Hort. Sci. 34:163-169.

Infante, R., E. Maganini, and B. Righetti. 1989. The role of light and CO_2 in optimizing the conditions for shoot proliferation of *Actinidia deliciosa* in vitro. Physiol. Plant. 77:191-195.

James, D.J., V.H. Knight, and I.J. Thurbon. 1980. Micropropagation of red raspberry and the influence of phloroglucinol. Sci. Hort. 12:313-319.

Jennings, D.L. 1988. Raspberries and blackberries: their breeding, diseases and growth. Academic Press, N.Y. pp. 227

Jones, J.B. 1986. Determining markets and market potential of horticultural crops. In: Tissue culture as a plant production system for horticultural crops. Zimmerman. R. H.(ed.). Martinus Nijhhoff Publisher, pp. 175-182.

Jones, O.P., and C.A. Webster. 1989. Improved rooting from conventional cuttings taken from micropropagated plants of *Pyrus communis* rootstocks. J. Hort. Sci. 64:429-434.

Karlsson, S., and B. Sveinbjornsson. 1981. Methodological comparison of photosynthetic rates measured by the $^{14}CO_2$ technique or infra-red gas analysis. Photosynthetica 15:447-452.

Keep. E. 1961. Autumn fruiting in raspberries. J. Hort. Sci. 36:174-185

Kozai, T. 1988a. Autotrophic (sugar free) tissue culture for promoting the growth of plantlets in vitro and for reducing biological contamination. Intnl. Symposium on Appl. Biotech. Small Industries Development in Developing Countries. Bangkok, Thailand.

Kozai, T. 1988b. High technology in protected cultivation: from environment control engineering point of point. Internl. Symposium High Tech. Protected Cult. Tokyo, 1988.

Kozai, T. 1989. Photoautotrophic micropropagation of some horticultural crops. 1989 ASHS annual meeting/program and abstracts 121.

Kozai, T. 1990. Growth of tobacco seedlings and plantlets in vitro as affected by in vitro environment. Environ. Control in Biol. 28:31-39. Text in Japanese with English summary.

Kozai, T. 1991. Micropropagation under photoautotrophic conditions, pp.447-469. In: Debergh P.C. and R.H. Zimmerman (eds.). Micropropagation technology and application. Kluwer Academic Publishers.

Kozai, T., and Y. Iwanami. 1988. Effects of CO_2 enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during the preparation stage. J. Japan. Soc. Hort. Sci. 57:279-288.

Kozai, T., Y. Iwanami, and K. Fujiwara. 1987. Effects of CO_2

enrichment on the plantlet growth during the multiplication stage. Plant Tissue Culture Let. 4:22-26 (Japanese with English summary).

Kozai, T., H. Oki, and K. Fujiwara. 1987. Effects of CO_2 enrichment and sucrose concentration under high photosynthetic photon fluxes on growth of tissue-culture Cymbidium plantlets during the preparation stage. In: Symposium Florizel 87: Plant propagation in horticultural industries. Ducate. (ed.). Arlon, Belgium. pp. 135-141.

Kozai, T., Y. Koyama, and I. Watanabe. 1988a. multiplication of potato plantlets in vitro with sugar free medium under high photosynthetic photon flux. Acta Hort. 230:121-126.

Kozai, T., C. Kubota, and I. Watanabe. 1988b. Effects of basal medium composition on the growth of carnation plantlets in auto- and mixo-trophic tissue culture. Acta Hort. 230:159-166.

Kozai, T., C. Kubota, and I. Watanabe. 1990a. The growth of carnation plantlets in vitro cultured photoautotrophically and photomixotrophically on different media. Envir. control in Biol. 28:21-27 (Japanese with English summary).

Kozai, T., H. Oki, and K. Fujiwara. 1990b. Photosynthetic characteristics of *Cymbidium* plantlet in vitro. Plant Cell, Tissue and Organ Culture 22:205-211.

Kozai, T., A. Takazawa, I. Watanabe, and J. Sugi. 1990c. Growth of tobacco seedlings and plantlets in vitro as affected by in vitro environment. Envir. Control in Biol.

28:31-39 (Japanese with English summary).

Kozai, T., K. Iwabuchi, K. Watanabe, and I. Wantanabe. 1991a. Photoautotrophic and photomixotrophic growth of strawberry plantlets in vitro and changes in nutrient composition of the medium. Plant Cell, Tissue and Organ Culture 25:107-115.

Kozai, T., N. Ohde, and C. Kubota. 1991b. Similarity of growth patterns between plantlets and seedlings of *Brassica campestris* L. under different in vitro environmental conditions. Plant Cell, Tissue and Organ Culture 24:181-186.

Laforge, F., Y. Desjardins, M.E.D. Graham, and A. Gosselin. 1990. Miniature growth chambers for study of environmental conditions in vitro. Can. J. Plant Sci. 70:825-836.

Laforge, F., C. Lussier, Y. Desjardins, and A. Gosselin. 1991. Effect of light intensity and CO_2 enrichment during in vitro rooting on subsequent growth of plantlets of strawberry, raspberry and asparagus in acclimatization. Sci. Hort. 47:259-269.

Lakso, A.N., B.I. Reisch, J. Mortensen, and M.H. Roberts. 1986. Carbon dioxide enrichment for stimulation of growth of in vitro-propagated grapvines after transfer from culture. J. Amer. Soc. Hort. Sci. 111:634-638.

Langford, P.J., and H. Wainwright. 1988. Influence of sucrose concentration on the photosynthetic ability of in vitro grown rose shoots. Acta Hort. 227:305-309.

Lawson, H.M. 1980. Recent research on cane vigor control in Scotland. Acta Hort. 112:151-156.

Lawson, H.M., and J.S. Wiseman. 1979. Effects of raspberry suckers, growing in the alleys between rows, on cane and fruit production in a non-cultivated plantation. Hort. Res. 19:63-74.

Lawson, H.M. and J.S. Wiseman. 1980. Effects of soil ridging and rotary cultivation on the growth and yield of a raspberry plantation. Hort. Res.20:33-42.

Leclerc, Y. 1989. Acclimatization, field performance and microtuberization of tissue cultured potato (*Solanum tuberosum* L.) cv. Russet Burbank. M.Sc. Thesis. McGill University, Montreal, Canada, pp.88.

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

Lewandowski, V.T. 1991. Rooting and acclimatization of micropropagated *Vitis labrusca* 'Delaware'. HortScience 26:586-589.

Liverani, A., D. Cobianchi, F.R. De Salvador, O. Insero, and A. Minguzzi. 1989. Field performance of two peach and nectarine cultivars propagated in vitro. Acta Hort. 254:41-46.

Long, S.P., and J.E. Hallgren. 1985. Measurement of CO_2 assimilation by plants in the field and the laboratory. in Techniques in bioproductivity and photosynthesis. Coombs, J. D.O. Hall, S.P. Long, and J.M.O. Scurlock, (eds.). N.Y. pp.

62-64

Mackerron, D.K.L. 1982. Growth and water use in the red raspberry (*Rubus idaeus* L.) I. growth and yield under different levels of soil moisture stress. J. Hort. Sci. 57:295-306.

Maene, L., and P.C. Debergh. 1987. Optimalisation of the transfer of tissue shoots to in vivo conditions. Acta Hort. 212:335-348.

Marcotrigiano, M., H.J. Swartz, S.E. Gray, D. Tokarcik, and J. Popenoe. 1984. The Effect of benzylaminopurine on the in vitro multiplication rate and subsequent field performance of tissue-culture propagated strawberry plants. Adv. Strawberry Prod. 3:23-25

Marin, J.A., and R. Gella. 1987. Acclimatization of micropropagated cherry rootstock 'Masto de montanana' (*Prunus cerasus* L.). Acta Hort. 212:603-606.

Marin, J.A., and R. Gella. 1988. Is desiccation the cause of the poor survival rate in the acclimatization of micropropagated *Prunus cerasus* L. Acta Hort. 230:105-112.

Marin, J.A., R. Gella., and M. Herrero. 1988. Stomatal structure and functioning as a response to environmental changes in acclimatized micropropagated *Prunus cerasus* L. Ann. Bot. 62:663-670.

Mason, D.T. 1981. A comparison of the hedgerow and stool systems for growing the red raspberry (*Rubus idaeus* L.) in relation to cane disease incidence and yield component

compensation. Hort. Res. 21:149-158.

McPheeters, K.D., R.M. Skirvin, and H.K. Hall. 1988. Brambles (*Rubus* spp.). In: Biotechnology in Agriculture and forestry. Vol. 6. Crops II. Bajaj, Y.P.S. (ed.). Springer-Verlag, Berlin. pp.104-123.

Meador, D.B. 1985. Impact of herbicides on field-planted, succulent, tissue-culture propagated, red raspberry plants. HortScience 20:526(abstr.).

Mohamed, F., H.J. Swartz. 1989. In vitro plant growth regulators effects on ex vitro behavior of strawberry plants. ASHS 1989 Annu. Mtg. Tulsa, Okla, Prog & Abstr. p.121 (abstr.).

Mohamed, F., H.J. Swartz, and J.G. Buta. 1991. The role of abscisic acid and plant growth regulators in tissue culture-induced rejuvenation of strawberry ex vitro. Plant Cell, Tissue and Organ Culture 25:75-84.

Moore, J.N., and J. Janick. 1975. Advances in fruit breeding. West Lafayette, Ind., Purdue University Press.

Moore, P.P, J.A. Robbins, and T.M. Sjulin. 1991. Field performance of 'Olympius' strawberry subclones. HortScience 26:192-194.

Morison, J.I.L. 1987. Intercellular CO_2 concentration and stomatal response to CO_2. In: Stomatal function. Zeiger, E., G.D. Farquhar, and I.R. Cowan (eds.). Standford Uuniversity Press. CA.

Murashige, T. 1974. Plant propagation through tissue culture.

Ann. Rev. Plant Physiol. 25:135-166.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Neal, J.C., P.M. Pritts, and A.F. Senesac. 1990. Evaluation of preemergent herbicide phytotoxicity to tissue culture -propagated 'Heritage' red raspberry. J. Amer. Soc. Hort. Sci. 115:416-422.

Nehrbas, S.R., and M. Pritts. 1988. Effect of pruning system on yield components of two summer-bearing raspberry cultivars. J. Amer. Soc. Hort. Sci. 113:314-321.

Nobel, P.S. 1991. Physiological and environmental plant physiology. Academic Press, Inc. N.Y. pp. 635.

Norton, R.A. 1980. Red raspberry primocane control research and practice in the Pacific Northwest. Acta Hort. 112:191-193.

Nova Scotia Department of Agriculture and Marketing. 1990. Rules and regulations governing the inspection and growing of certified raspberry plants.

Ourecky, D.K. 1975. Brambles. In Advances in fruit breeding eds. Janick, J., and J.N. Moore. Purdue University Press. pp. 71-97.

Pierik, R.L.M. 1988. Handicaps for the large scale commercial application of micropropagation. Acta Hort. 230:63-71.

Pliego-Alfaro, M., and T. Murashige. 1987. Possible rejuvenation of adult avocado by graftage onto juvenile

rootstock in vitro. HortScience 22:1321-1324.

Preece, J.E., and E.G. Sutter. 1991. Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh P.C. and R.H. Zimmerman (eds.) Micropropagation technology and application. Kluwer Academic Publishers. pp. 71-94.

Putz, C. 1971. Obtention de framboisiers (var. Bois Blanc) sans virus par la technique des culture de meristemes. Ann. Phyto. pathol. 3:493-501.

Pyott, J.L., and R.H. Converse. 1981. In vitro propagation of heat-treated red raspberry clones. HortScience 16:308-309.

Quak, F. 1977. Meristem culture and virus-free plants. In: Applied and fundamental aspects of plant cell, tissues and organ culture. J. Reinert, and Y.P.S. Bajaj (eds.). Berlin, Springer-Verlag, pp. 598-615.

Quebec Ministere de l'Agriculture, des Pecheries et de l'Alimentation. 1992. Certification program of strawberry and raspberry. pp. 20.

Rahman, M.A., and J. Blake. 1988. The effects of medium composition and culture conditions on in vitro rooting and ex vitro establishment of jackfruit (*Artocarpus heterophyllus* Lam.). Plant Cell Tissue and Organ Culture 13:189-200.

Roberts, A.V., and E.F. Smith. 1990. The preparation in vitro of chrysanthemum for transplantation to soil. 1. Protection of roots by cellulose plugs. Plant Cell Tissue Organ Culture

21:129-132.

Robinson, S.P., W.J.R. Grant, and B.R. Loveys. 1988. Stomatal limitation of photosynthesis in abscisic acid-treated and in water-stressed leaves measured at elevated CO_2. Aust. J. Plant Physiol. 15:495-503.

Rosati, P., and D. Gaggioli. 1987. Field performance of micropropagated peach rootstocks and scion cultivars of sour cherry and apple. Acta Hort. 212:379-390.

Rosati, P., and D. Gaggioli, and L. Giunchi. 1986. Genetic stability of micropropagated loganberry plants. J. Hort. Sci. 61:33-41.

Rowe, J.W. 1986. New technologies in plant tissue culture. In: Tissue culture as a plant production system for horticultural crops Zimmerman. R.H.(Ed.). Martinus Nijhhoff Publisher PP 35-51.

Sagawa, Y., and J.T. Kunisaki. 1990. Micropropagation of floriculture crops. In: Sharp, W.R., Evans, D.A., Ammirato, P.V, and Yamada, Y (eds.). Hand book of plant cell culture. Vol. 5. Macmillan Publishing Company.

SAS Institute Inc. 1985. SAS user's guide: statistics. 5th ed. Cary N.C. U.S.A. pp. 956.

Scorza, R. 1982. In vitro flowering. Hort. Rev. 4:106-127.

Scott, D.H., G.J. Galletta, and H.J. Swartz. 1985. Tissue culture as an aid in the propagation of 'Tribute' everbearing strawberry. Adv. strawberry Prod. 4:59-60.

Sestak, Z., J. Catsky, and P.G. Jarvis. 1971. Plant

photosynthetic production: manual of methods. Dr. W.Junk N.V. Publishers the Hague.

Shackel, K.A., V. Novello, E.G. Sutter. 1990. Stomatal function and cuticular conductance in while tissue-cultured apple shoots. J. Amer. Soc. Hort. Sci. 115:468-472.

Shchelkunova, S.E., and Y.G. Popov. 1970. Production of virus-free raspberry plants from cultures of isolated apexes. Soviet plant physiology, Engl. Trans. Fisiol. Rast. 17:618-621.

Shimada, N., F. Tanaka, and T. Kozai. 1988. Effects of low O_2 concentration on net photosynthesis of C_3 plantlets in vitro. Acta Hort. 230:171-175.

Short, K.C., J. Warburton, and A.V. Roberts. 1987. In vitro hardening of cultured cauliflower and chrysanthemum plantlets to humidity. Acta Hort. 212:329-334.

Shoemaker, J.S. 1978. Small fruit culture. The Avi publishing company, Inc., Westport, Connecticut.

Shoemaker, N.P., H.J. Swartz, and G.J. Gelletta. 1985. Cultivar dependent variation in pathogen resistance due to tissue culture-propagation of strawberries. HortScience 20:253-254.

Skirvin, R.M. 1976. Tissue culture-induced variation in scented *Pelargonium* spp. J. Amer. Soc. Hort. Sci. 101:281-290.

Skirvin, R.M. 1978. Natural and induced variation in tissue culture. Euphytica 27:241-266.

Smith, E.F., A.V. Roberts, and J. Mottley. 1990a. The preparation in vitro of chrysanthemum for transplantation to soil. 2. improved resistance to desiccation conferred by paclobutrazol. Plant Cell, Tissue and Organ Culture 21:133-140.

Smith, E.F., A.V. Roberts, and J. Mottley. 1990b. The preparation in vitro of chrysanthemum for transplantation to soil, 3: improved resistance to desiccation conferred by reduced humidity. Plant Cell, Tissue and Organ Culture 21:141-145.

Smith, E.F., A.V. Roberts, J. Mottley, and S. Dennes. 1991. The preparation in vitro of chrysanthemum for transplantation to soil, 4: the effects of eleven growth retardants on wilting. Plant Cell, Tissue and Organ Culture 27:309-313.

Smith, M.A.L., J.P. Palta, and B.H. McCown. 1986. Comparative anatomy and physiology of microcultured, seedling, and greenhouse-grown Asian white birch. J. Amer. Soc. Hort. Sci. 111:437-442.

Smith, E.F., I. Gribaudo, A.V. Roberts, and J. Mottley. 1992. Pactobutrazol and reduced humidity improve resistance top wilting of micropropagated grapevine. HortScience 27:111-113.

Snedecor, G.W., and W.G. Cochran. 1980. Statistical methods. 7th Ed. Iowa State University Press.

Snir, I. 1981. Micropropagation of red raspberry. Sci. Hort.

14:139-143.

Snir, I. 1988. Red raspberry (*Rubus idaeus*). In Biotechnology in Agriculture and forestry. Vol. 6. Crops II. Bajaj, Y.P.S. (ed.). Springer-Verlag, Berlin. pp.124-141.

Sobczykiewz, D. 1987. Mass production of raspberry plants by meristem culture. Acta Hort. 212:677-681.

Stace-Smith, R. 1984. Red raspberry viruses disease in North America. Plant Disease 68:274-279.

Statistics Canada. 1989. Fruit and vegetable production. Catologue #:22-003.

Steel, R.G.D., and J.H. Torrie. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill Book company. N.Y.

Styer, D.J., and C.K. Chin. 1983. Meristem and shoot-tip culture for propagation, pathogen elimination and germplasm preservation. In: Hort. Rev. Vol 5, J. Janick (ed.). Westport, Conn.: AVI Publ. Co. pp. 221-227.

Sutter, E. 1978. Epicuticular wax and cuticle formation in meristem regenerated plants of carnation. HortScience 13:348(abstr.).

Sutter, E. 1982. Presence of epicuticular wax on several species of plants grown in culture, greenhouse and in growth chamber. HortScience 17:488 (abstr.).

Sutter, E. 1984. Chemical composition of epicuticular wax in cabbage plants grown in vitro. Can. J. Bot. 74-77.

Sutter, E. 1985. Morphological, physical, and chemical

characteristics of epicuticular wax on ornamental plants regenerated in vitro. Ann. Bot. 55:321-329.

Sutter. E. 1988. Stomatal and cuticular water loss from apple, cherry and sweetgum plants after removal from in vitro culture. J. Amer. Soc. Hort. Sci. 113:234-238.

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

Sutter, E., and R.W. Langhans. 1982. Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot-tip culture. Can. J. Bot. 60: 2896-2902.

Swartz, H.J. 1991. Post culture behavior: genetic and epigenetic effects and related problems, pp.95-122. In: Debergh P.C. and R.H. Zimmerman (eds.) Micropropagation technology and application. Kluwer Academic Publishers.

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

Swartz, H.J., G.J. Galletta, and R.H. Zimmerman. 1983. Field performance and phenotypic stability of tissue culture-propagated thornless blackberries. J. Amer. Soc. Hort. Sci. 108:285-290.

Swartz, H.J., and J.T. Lindstrom. 1986. Small fruit and grape tissue culture from 1980-1985 commercialization of the

technique. In: Tissue culture as a plant production system for horticultural crops. Zimmerman. R. H.(Ed.). Martinus Nijhhoff Publisher PP 201-220.

Theiler-Hedtrich, R., and H. Wolfensberger. 1987. Comparison of plants and yield characters of in vitro and normal propagated strawberry plants. Acta Hort. 212:445-448.

Tisdall, L. 1989. Acclimatization of micropropagated 'Silvan' blackberry. M.Sc. Thesis, McGill University, Montreal, Canada. pp. 94.

Tisdall, L., and D.J. Donnelly. 1988. A special clearing and staining method for tissue-cultured plants and greenhouse grown leaves. HortScience 23:1059-1061.

Torre, L.C., and B.H. Barritt. 1979. Red raspberry establishment from root cutting. J. Amer. Soc. Hort. Sci. 104:28-31.

Trinka, D., and M. Pritts. 1990. Tissue-cultured red raspberry plant establishment. HortScience 25:1162(abstrt.).

Van Huystee, R.B. 1977. Porphyrin metabolism in peanut cells cultured in sucrose containing medium. Acta Hort. 78:83-87.

Vanderschaeghe, A.M., and P.C. Debergh. 1988. Automation of tissue culture manipulations in the final stages. Acta Hort. 227:399-401.

Vasil, I. 1991. Cell culture and somatic cell genetics of plants. Vol. 8, Academic Press, N.Y. pp.267.

Vasliakakis, M.D., and M.N. Dana. 1978. Influence of primocane inflorescence removal on number inflorescences and suckers

in 'Heritage' red raspberry. HortScience 13:700-701.

Vertesy, J. 1979. Experiments on the production of viruses free raspberry propagation materials by meristem culture. Acta Hort. 95:77-81.

Wainwright, H., and A.W. Flegmann. 1984. The influence of light on the micropropagation of blackcurrant. J. Hort Sci. 59:387-393.

Walker, P.N, C.W. Heuser, and P.H. Heinemann. 1988. Micropropagation: studies of gaseous environments. Acta Hort. 230: 145-151.

Wardle, K., and K.C. Short. 1983. Stomatal response of in vitro cultured plantlets: I. Responses in epidermal strips of *Chrysanthemum* to environmental factors and growth regulators. Biochem. Physiol. Pflanzen 178:619-624.

Wardle, K., E.B. Dobbs, and K.C. Short. 1983. In vitro acclimatization of aseptically cultured plants to humidity. J. Amer. Soc. Hort. Sci. 108:386-9.

Wardle, K., A. Quinlan, and I. Simpkins. 1979. Abscisic acid and the regulation of water loss in plantlets of *Brassica oleracea* L. var. botritis regenerated through apical meristem culture. Ann. Bot. 43:745-52.

Wardle, K., V. Dalson, I. Simpkins, and K.C. Short. 1983. Redistribution of Rubidium in plants of *Chrysanthemum morifolium* Ram. cv, Snowdon derived from tissue culture and transferred to soil. Ann Bot. 51:261-264.

Webster, A.D., V.D. Oehl, J.E. Jackson, and O.P. Jones. 1985.

The orchard establishment, growth and precocity of four micropropagated apple scion cultivars. J. Hort. Sci. 60:169-180.

Webster, A.D., T.R. Sparks, and A. Belcher. 1986. The influence of micropropagation and chemical mutagens on the growth and precocity of 'Cox's Orange Pippin' and 'Brambley's Seedling' apple. Acta Hort. 180:25-34.

Welander, M. 1985. In vitro culture of raspberry (*Rubus idaeus*) for mass propagation. J. Hort. Sci. 60:493-499.

Wetherell, D.F. 1982. Introduction to in vitro propagation. Avery Publishing Group Inc. U.S.A. 73 pp.

Wetmore, R.H., and J.P. Rier. 1963. Experimental induction of vascular tissue in callus of antiosperms. Amer. J. Bot. 50:418-430.

Wetzstein, H.Y., and H.E. Sommer. 1981. Transmission electron microscopy of cultured and acclimatized *Liquidambar styraciflua*. HortScience 16:405 (abstr.).

Wetzstein, H.Y., and H.E. Sommer. 1982. Leaf anatomy of tissue-cultured *Liquidambar styraciflua* (Hamamelidaceae) during acclimatization. Amer. J. Bot. 69: 1579-1586.

Wetzstein, H.Y., and H.E. Sommer. 1983. Scanning electron microscopy of in vitro-cultured *Liquidambar styraciflua* plantlets during acclimatization. J. Amer. Soc. Hort. Sci. 108:475-480.

Wetzstein, H.Y., H.E. Sommer, C.L. Brown, and H.M. Vines. 1981. Anatomical changes in tissue cultured sweet gum leaves

during the hardening-off period. HortScience 16:290 (abstr.).

Whitney, G.G. 1982. The productivity and carbohydrate economy of a developing stands of *Rubus idaeus*. Can. J. Bot. 2697-2703.

Williams, I.H. 1959a. Effects of environment on *Rubus idaeus* L. II. Field observations on the variety Malling Promise. J. Hort. Sci. 34:170-175.

Williams, I.H. 1959b. Effects of environment on *Rubus idaeus* L. III. Growth and dormancy of young shoots. J. Hort. Sci. 34:210-218.

Williams, I.H. 1959c. Effects of environment on *Rubus idaeus* L. IV. Flower initiation and development of the inflorescence. J. Hort. Sci. 34:219-228.

Williamson, B., H.M. Lawson, J.A.T. Woodford, A.J. Hargreaves, J.S. Wiseman, and S.C. Gordon. 1979. Vigour control, an integrated approach to cane, pest and disease management in red raspberry (*Rubus idaeus* L.). Ann. Appl. Biol. 92:359-368.

Wright, C.J., and P.D. Waister. 1982a. Within-plant competition in the red raspberry. I. Primocane growth. J. Hort. Sci. 57:437-442.

Wright, C.J., and P.D. Waister. 1982b. Within-plant competition in the red raspberry. II. Fruiting cane growth. J. Hort. Sci. 57:443-448.

Yue, D., Y. Desjardins, M. Lamarre, and A. Gosselin. 1992.

Photosynthesis and transpiration of in vitro cultured asparagus plantlets. Sci. Hort. 49:9-16.

Zimmerman, R.H. 1986. Propagation of fruit, nut, and vegetable crops - overview, pp 183-200. In: Zimmerman, R.H., Griesbach, R.J., Hammerschlag, F.A., and Lawson, R.H., (eds.). Tissue culture as a plant production system for horticultural crops. Martinus Nijhoff Publishers.

Zimmerman, R.H. 1988. Micropropagation of woody plants: post tissue culture aspects. Acta Hort. 227:489-499.

Zimmerman, R.H. 1991. Response of micropropagated apple trees to field establishment procedures. HortScience. 26:1364-1365.

Zimmerman, R.H., and J.B. Jones. 1991. Commercial micropropagation in North America. In: Debergh P.C. and R.H. Zimmerman (eds.). Micropropagation technology and application. Kluwer Academic Publishers. pp.173-180.

Zimmerman, R.H., and S.S. Miller. 1991. Orchard growth and fruiting of micropropagated apple trees. J. Amer. Soc. Hort. Sci. 116:780-785.

Zimmerman, R.H., and G.L. Steffens. 1989. Management of self-rooted tissue-cultured apple tress: I. orchard establishment and early growth. Acta Hort. 239:117-124.

Ziv, M. 1986. In vitro hardening and acclimatization of tissue cultured plants. In: Plant tissue culture and its agricultural implications. Withers. L.A., and P.G. Alderson, (eds.). London. pp. 187-196.

Ziv, M. 1991. Vitrification:morphological and physiological disorders of in vitro plants. In: Debergh P.C. and R.H. Zimmerman (eds.). Micropropagation technology and application. Kluwer Academic Publishers. pp. 45-70.

Ziv, M., A. Schwartz, and D. Felminger. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plantlets propagated in vitro; implication for hardening. Plant Sci.52:127-134.

Ziv, M., G. Meir, and A.H. Halevy. 1983. Factors influencing the production of hardened glaucous carnation plantlets in vitro. Plant Cell, Tissue and Organ Culture 2:55-65.