EPIGENETIC VARIATION IN THE ANATOMY AND PHYSIOLOGY OF TISSUE CULTURED RED RASPBERRY PLANTLETS AND TRANSPLANTS

by

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Epigenetic Variation in the Anatomy and Physiology of

Tissue Cultured Red Raspberry Plantlets and Transplants

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ABSTRACT

The intent of this study of the anatomical and physiological changes found in plantlets and transplants of a cloned Rubus idaeus L. selection was to provide objective assessment of medium and environmental parameters prior to transplant and determine some of the conditions that influence acclimatization of transplants.

Anatomy and leaf surface features of plantlets and persistent and new growth of transplants were examined. Growth in culture promoted small, thin leaves with less compact chlorenchyma tissue, an altered palisade cell shape and many other differences compared to controls. Stomatal and trichome numbers and distribution were affected as was stomatal function. Collenchyma and sclerenchyma support tissues and secondary wall development were inhibited in vitro in all organs. New growth of transplants was intermediate (transitional) between culture and control plants and affected by the transplant light intensity.

Data was collected on gas exchange, a variety of leaf parameters including photosynthetic pigment content, fresh/dry weight, percentage water content, gram dry weight/area and total plantlet leaf area. Starch storage capacity, chlorophyll a fluorescence induction and oxygen evolution rates were measured in cultured and control plants. Cultured plantlets demonstrated relatively low levels of CO2 uptake compared to control plant rates, while transplants had intermediate rates. The succession of new leaves of transplants improved anatomically and in $^{14}CO2$

uptake ability, both of which were influenced by light intensity. Pigment content, fresh/dry weight, percentage water content and gram dry weight/area were all affected by culture. Persistent leaves of transplants did not increase in size with time but did accumulate dry matter at the higher light intensities; probably at the expense of the new leaves. Cultured plantlet leaves showed decreased starch storage capacity and reduced oxygen evolution rates compared to controls. Spent medium was found to have potential as an analytical tool in culture evaluation and improvement.

Anatomical and physiological function data are summarized and interpreted based on an epigenetic change concept that includes acclimatization phenomena. Anatomical, physiological and medium tests are considered in medium and environmental parameter evaluation in plant tissue culture.

DEDICATION

This thesis is dedicated to people who work with and study tissue cultured plants.

QUOTATION

If a bud be torn from the branch of a tree and cut out and planted in the earth with a glass cup inverted over it to prevent the exhalation from being at first greater than its power of absorption; or if it be inserted into the bark of another tree, it will grow, and become a plant in every respect like its parent. This evinces that every bud of a tree is an individual vegetable being; and that a tree therefore is a family or swarm of individual plants, like the polypus, with its growing young out of its sides, or like the branching cells of the coral insect... the shoot is a succession of individual vegetable members. (Erasmus Darwin, 1800; cited by White, 1979. page 109.)

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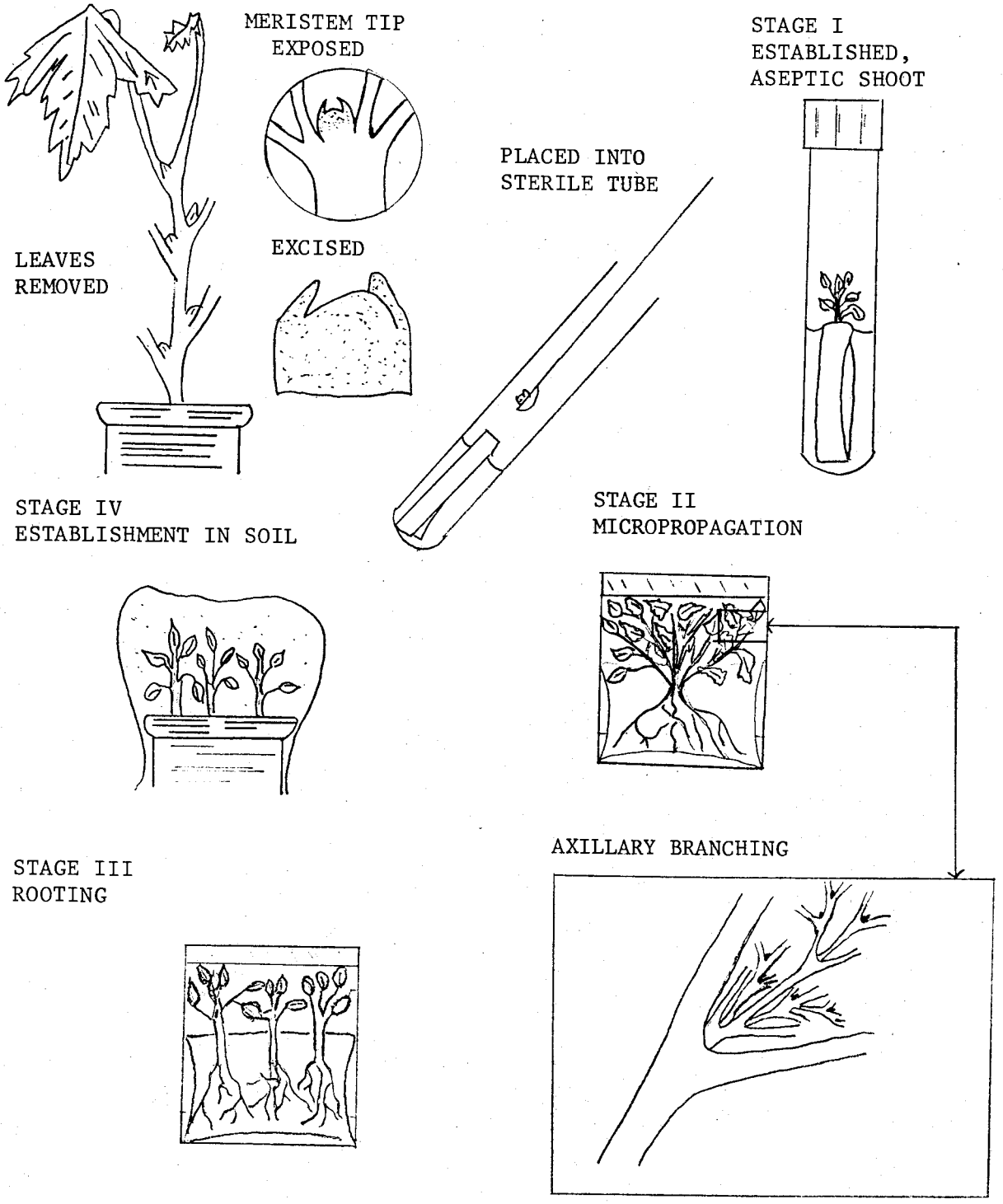
A. General Introduction

Currently the most advanced aspect of plant tissue culture (Conger, 1981) with the greatest practical application (Murashige, 1977a, 1978a) is the rapid multiplication or clonal propagation of select genotypes. Of the tissue culture techniques involved in asexual multiplication of plants, a preferred method has been that of enhanced axillary branching in vitro (Fig. 1, inset). While this method is relatively slow, it has broad applicability and produces less genetic variation than adventive processes (Murashige, 1977b). Axillary branches (shoots) are stimulated to grow by cytokinin-containing nutrient medium. These branches are cut apart and either subcultured to new medium for continued shoot proliferation or rooted. Murashige (1978b) describes four sequential steps or stages for in vitro propagation, although fewer or greater number of steps are sometimes used (Fig. 1). It is not unusual to realize through this process million-fold increases in clonal plant numbers over traditional propagative means. In stage I a stem tip or nodal bud explant is established aseptically in vitro. In stage II clonal increase occurs. This stage can be repeated many times by subculture of newly emerged shoots to new medium. For some cultivars direct transfer from stage II to soil is possible. For many other plants, an additional culture step (stage III), during which the shoots are individually rooted, improves survival upon transplant to soil (stage IV).

Figure 1. The four sequential stages of tissue culture propagation; inset, axillary branching.



The current approaches to media formulation and decisions involving optimal environmental parameters to sustain plant growth are based on a limited number of criteria which include number of survivors (stage I and IV), number of axillary shoots per culture cycle (stage II), "greenness", "vigour" and "hardiness" (stage III and IV), and "normalacy" and "trueness-to-type" (stage IV). Though the majority of tissue culturists employ the Murashige and Skoog (1962) basal medium formulation, developed for tobacco callus growth, they do not use fresh or dry weight tissue yields, as these authors did, in medium assessment. While media formulation need no longer be the empirical procedure it once was, even relatively sophisticated approaches such as that elaborated by deFossard et al. (1974), a proponent of the multifactorial approach, affect no suggestion as to how best to evaluate the progress of cultured plantlets. Evaluation is done intuitively a) in a manner that reflects success or failure: it survived-it didn't; it grew-it didn't; it produced axillary branches-it never branched; it rooted-it didn't; it transplanted-it died b) while success or failure terminology is often applied to stages I, III and IV, criteria may extend to shoot or plant size as well as numbers. Length may serve as a determinant to transfer stage I propagules to stage II culture conditions for rapid multiplication and shoot length and numbers send stage II propagules to repeat subculture or to stage III or IV for rooting. Plant height is sometimes used to evaluate stage IV transplants after a period of establishment in

soil (Miller and Murashige, 1976) c)the general state of health of the plantlets is sometimes described, usually in subjective terms such as "chlorotic", "watery", "vigorous" and the like. A prime objective of this thesis is to suggest the replacement of this subjective and inadequate terminology with descriptive anatomical and/or functional criteria.

Decisions to transfer plantlets to new medium or to soil should be based on quantitative performance criteria. The primary objective of this study was to compare stage III and IV plantlets with control plants in a search for anatomical markers and physiological tests to use as criteria to evaluate or measure performance. In this process spent medium was examined as an indicator of plantlet performance *in vitro*. The corollaries were to demonstrate that anatomical and/or physiological criteria or medium analysis used to measure performance or functional competence *in vitro* facilitate decisions involving changes in the culture environment. A further aim was to investigate some of the features of acclimatization in transplants.

Red raspberry (*Rubus idaeus* L.) was selected for use in these experiments because of its economic importance in British Columbia and the author's familiarity with the culture of *Rubus* species. In 1983 British Columbia had approximately 2,500 hectares devoted to red raspberry with every indication of continued expansion. These had the highest yields per unit area of any region in the world (Daubeny, 1983). For a complete

description of the culture of Rubus species and an in depth review of the literature concerning their culture see Donnelly (1980) and Donnelly, Stace-Smith and Mellor (1980).

The acclimatization of cultured plants to soil has received relatively little attention (Sutter and Langhans, 1979; Wetzstein and Sommer, 1982, 1983) though it remains a major obstacle in commercial tissue culture micropropagation (Anderson, 1980), and is often the limiting factor in its success (Poole and Conover, 1983). When plantlets are transplanted from culture to soil conditions they wilt rapidly, desiccate readily and may die as a result of the changes in relative humidity. For some plants tenting and/or misting reduces or eliminates transplant losses, for others such as carnation (50% losses; Earle and Langhans, 1975; 30-90% losses; Sutter and Langhans, 1979) and thornless blackberry (40% losses; Broome and Zimmerman, 1978) these tactics are not sufficient to ensure survival of all of the regenerated plants. Imperative to an understanding of the acclimatization process, is that it be related to both structural anatomy and physiological processes of plantlets undergoing environmental change. The terms acclimation and acclimatization describe the process of adaptation of an organism to environmental change; the acclimation process is regulated by nature; the acclimatization process is regulated by man. These definitions were accepted by the 1977 Environmental Conditioning Symposium (Brainerd and Fuchigami, 1981). In this thesis a cautious interpretation of

the term acclimatized has been employed. It is frequently used in the literature to describe transplants at various intervals after transfer to soil. These often differ from control plants in various ways. More correctly, these transplants were undergoing the process of acclimatization, and should not be termed acclimatized until indistinguishable from controls. The word transplant has been substituted for acclimatized plant in many instances to avoid confusion. In select cases it has been retained to indicate control levels of the specific item under investigation (such as acquisition of normal stomatal function). It should however be kept in mind that this is acclimatization only in a limited sense; it does not describe the multiplicity of untested anatomical and physiological characteristics that are affected by environmental conditions.

In the literature the primary concerns have been with water loss of transplants due to deficiencies of a)epicuticular or cuticular wax, b)stomatal control or c)foliar anatomy.

a) Low survival rate of transplants has been attributed to excess cuticular transpiration because of lack of epicuticular wax in cauliflower (Grout, 1975; Grout and Aston, 1977a) and carnation (Sutter and Langhans, 1979). Grout (1975) reported that epicuticular wax developed on the regenerated foliage leaves of cauliflower (Brassica oleracea var botrytis 'Currawong') plantlets only after transplantation. After 10 days in soil persistent leaves apparently resembled control leaves as did new leaves formed after transplanting. Grout and Aston

(1977a) suggested that excessive water loss of regenerants (B. oleracea var botrytis 'Armado Tardo') was caused by severely reduced epicuticular wax formation in vitro due to the high humidity of culture. Transplanted carnation (Dianthus caryophyllus L. 'White Pike's Peak') developed irregular epicuticular wax deposits during 2.5 weeks of hardening in the greenhouse. Elevated light intensity (10,000 lux) in stage III culture did not affect the amount of leaf surface wax of transplants (Sutter and Langhans, 1979). Baker (1974) showed that increases in radiant energy or decreases in temperature or humidity could influence the amount, as well as the conformation and distribution of leaf surface wax. However, he pointed out that the proportional increase of wax with increasing light intensity is very little at elevated relative humidity. No differences were detected in the surface wax structure of cultured sweetgum (Liquidambar styraciflua) plants (Wetzstein and Sommer, 1983).

b) In transplanted plum (Prunus institia L. 'Pixy') water loss was shown to be primarily abaxial and either cuticular from lack of epicuticular wax, or stomatal (Fuchigami, Cheng and Soeldner, 1981). Stomatal frequencies were less in 'Pixy' plum plantlet leaves than in either transplants or controls (Brainerd, Fuchigami, Kwiatkowsky and Clark, 1981). Wetzstein and Sommer (1983) reported the surprising findings that stomatal frequencies were greater in sweetgum plantlets than in controls and stomatal length averaged 17 um, slightly longer than the 14

um of the controls and transplants. Wetzstein and Sommer (1982, 1983) showed that leaves of sweetgum plantlets in culture had raised circular stomata. Guard cells protruded from the abaxial surface *in vitro* unlike those of control leaves. The adaxial epidermal cells were oval in cultured leaves. The epidermal cells had irregular undulations in their anticlinal walls. Leaves from transplants, as in the control plants, had depressed ellipsoid stomata; the guard cells did not protrude like those of cultured leaves. Epidermal cells retained their irregular oval shape in transplants, with wavy anticlinal walls, similar to leaves grown in culture. Epidermal cells were isodiametric (cubical) in control plant leaves with regular and evenly arranged anticlinal walls. Brainerd and Fuchigami (1981) showed that acclimatization to low relative humidity could be monitored using leaf relative water content and percentage stomatal closure. They demonstrated using 'Pixy' plum that leaf water loss was directly related to stomatal closure, and acclimatization to low relative humidity involved the development of an accelerated stomatal response. Leaves of cultured apple (*Malus domestica* (Borkh.) 'Mac 9') were found to have no nocturnal stomatal closure pattern, nor did they close in response to 4 hours darkness, 4 hours 1 M mannitol induced water stress, 1 hour 10^{-4} M abscisic acid treatment nor 1 hour of 0.12% CO_2, until the transplants had acclimatized. Stomatal response for plum and apple transplants approximated that of control plants within 4-5 days at 30-40% relative humidity

(Brainerd and Fuchigami, 1981, 1982). Stomata of red raspberry plantlets did not close at night, nor in response to several hours of 1 M abscisic acid or elevated CO2 treatment (Donnelly and Vidaver, unpublished). From x-ray micro-probe analysis Wardle, Quinlan and Simpkins (1979) suggested that lower K:Na concentration ratios of *in vitro* cauliflower contributed to the poor stomatal closure mechanism.

Wardle, Dobbs and Short (1983) attempted to grow chrysanthemum (*Chrysanthemum* x *morifolium* Ramat. 'Snowdon'), and cauliflower (*B. oleracea* L. var *botrytis*) plantlets *in vitro* with a layer of lanolin covering the medium, with or without a packet of silica gel enclosed in the culture container, to further control the relative humidity. The surface layer of lanolin brought the relative humidity down from higher than 90% to 33% which was accompanied by retardation of plantlet growth. The addition of silica gel reduced the relative humidity to 10-20%, with high mortality. Reduced humidity was reported to affect stomatal development by increasing the number of immature stomata and reducing stomatal aperature and resulted in increased leaf surface wax. Both reduced stomatal aperature and increased leaf surface wax caused a decrease in the rate of water loss in detached leaves. It was suggested, but not shown, that if the lanolin layer were to be applied very late in culture, the growth retarding effects of reduced relative humidity would be offset by increased survival on transplantation.

c) Grout and Aston (1978) described a modified leaf anatomy of cauliflower plantlets in culture with absent or ill defined palisade cells. Brainerd, Fuchigami, Kwiatkowski and Clark (1981) observed that palisade cell length and tissue depth was less in 'Pixy' plum plantlets than transplants, and these were less than in control plants. Studies by Manning, Miller and Teare (1977) with pea plants grown under conditions of controlled relative humidity showed that excessive moisture prevented cell enlargement and elongation. They found that leaf blade thickness was severely affected and that palisade and spongy mesophyll cells were much smaller under conditions of moisture stress (very high or low relative humidity). Wetzstein and Sommer (1982) examined leaf anatomy of sweetgum in culture, 45 days after transplant and in control plants. Leaves of plantlets in culture had lower mesophyll cell density. Reportedly no palisade layer differentiated, unlike control leaves which had a well defined palisade and spongy parenchyma. Mesophyll cells in cultured leaves were extremely lacunose, with parietal cytoplasm and prominent nuclei. Leaves in vitro had flattened plastids with irregularly arranged internal membrane systems. These lacked organization into definite grana and stroma lamella and possessed relatively few starch granules. Leaves of transplants were intermediate in their characteristics (Wetzstein and Sommer, 1982). The mesophyll tissue showed some differentiation into palisade and spongy parenchyma and the adaxial mesophyll was denser and more elongate than in plantlets

from culture. Mesophyll cells had a greater amount of cytoplasm and some large vacuoles. The percentage mesophyll air space was reduced. These leaves had chloroplasts that were lens shaped and less flattened than those from either culture or field conditions. They had well developed grana and frequent starch granules. Absence of internal membrane development and reduced numbers of starch granules within the plastids of leaves in vitro suggested a reduced photosynthetic ability, as did the lack of palisade parenchyma in these plants (Wetzstein and Sommer, 1982).

Grout and Aston (1977b), in one of the few studies on the physiology of micropropagated plantlets, reported that cauliflower plantlets had less chlorophyll than seedlings of comparable age and that chloroplasts from in vitro plantlets had reduced Hill activity. Cauliflower plantlets, transplanted after 6 weeks in vitro apparently did not demonstrate net CO2 uptake in the light. Cauliflower regenerants lost most of their leaves immediately after transplant. This was felt by the authors to be of survival advantage since it reduced the tissue to be maintained from stored carbon and low levels of photosynthesis (Grout and Aston, 1978). In the second week of the hardening program new leaves, apparently with full photosynthetic ability, were produced by the transplants.

B. Materials and General Methods

The red raspberry plantlets employed in these experiments were a clone derived from one meristem tip, from a new selection developed by the British Columbia red raspberry breeding program, a 'Haida' x 'Canby' cross (BC72-1-7).

Plantlets were maintained in 500 ml jars with plastic covers containing a 3 cm wide filter paper support strip around the circumference of the jar, and 30 ml of modified red raspberry rooting medium (Donnelly, Stace-Smith and Mellor, 1980) in that 25 g/L glucose replaced 30 g/L sucrose. Plantlets were subdivided and subcultured at 4 week intervals in new medium or transferred to soil. Cultures were maintained at 5 light intensities, from 2,000 to 6,000 lux.

Plantlets were rinsed of medium as they were removed from culture and their leaves were tagged with coloured thread. After leaves were tagged 6 transplants were placed per 13 x 17 cm flat with sterilized greenhouse potting soil and a slow release fertilizer. The transplants were kept under conditions of elevated relative humidity by closing the flats with transparent covers and misting them regularly. Cultures and transplants were incubated under controlled temperature (27 ±2 °C) and lighting (16 hour photoperiod with 3:1 cool white:warm white fluorescent lighting). Light unit conversion from lux, utilized throughout this thesis, to uEinsteins/m^2/second is accomplished by multiplying Klux by 12.6.

Details of specific techniques are provided in appropriate sections of the thesis.

C. Epigenetic Variation in the Anatomy and Physiology of Tissue Cultured Red Raspberry Plantlets and Transplants

I. Leaf Anatomy of Red Raspberry in Culture and Transferred to Soil

Introduction

Despite increased use of tissue culture for the clonal propagation of horticultural crops (Murashige, 1977,1978) poor survival rates occur during the transfer of plantlets from *in vitro* conditions to soil environments (Boxus and Quoirin, 1977; Broome and Zimmerman, 1978; Earle and Langhans, 1975; Hasegawa, 1979; Skirvin and Chu, 1979). Recent investigations have shown that transplant shock is due primarily to water stress which can be combatted successfully for some plant species with high humidity after transplanting (Jones and Hopgood, 1979; Rosati, Marino and Swierczewski, 1980). Poor epicuticular and cuticular wax formation (Fuchigami, Cheng and Soeldner, 1981; Grout and Aston, 1977; Sutter and Langhans, 1979) and reduced stomatal control compared to greenhouse acclimatized plants (Brainerd and Fuchigami, 1981; Sutter and Langhans, 1979) contribute to desiccation of transplanted plantlets. Brainerd, Fuchigami, Kwaitkowski and Clark (1981) indicated that the rapidity of injury due to water stress was the result of small palisade cells, large intercellular spaces and low stomatal frequency of *in vitro* plantlet leaves.

This study characterizes some features of the internal and external leaf structure of red raspberry plantlets grown in aseptic culture before and after transfer to soil, and evaluates both plantlet and leaf survival under controlled environmental conditions.

Materials and Methods

Leaf samples were taken from plantlets cultured at 5 light intensities (2,000-6,000 lux) and transplants from 3,000 lux culture transferred to 3,000, 6,000 and 9,000 lux in soil. Leaves were collected from the lowest portions of aseptically cultured plantlets, and from both tagged (from culture) and new leaves formed after plantlets were transferred to soil. Young and mature leaves were collected from greenhouse and field grown plants for comparison. These plants were 1-2 years old and subject to ambient climatic conditions except for supplemental fertilizing and watering.

Anatomical features were examined from leaf samples cut into small sections, fixed in formalin-acetic acid-alcohol, dehydrated in an ethanol series, embedded in wax, sectioned (8 um), stained in safranin, counterstained in fast green and mounted in permount (Sass, 1958). Palisade and upper and lower epidermal cell lengths and widths were measured for at least 10 cells per leaf. Cell width was defined in the adaxial-abaxial plane. Calculations were made of average width of epidermal cell

(upper and lower), palisade tissue and mesophyll tissue as a percentage of average total leaf width. This effectively corrected for differences in leaf size.

Leaf surface structures were examined microscopically on leaves cleared by overnight-soaking in 1-4% chloral hydrate, bleached in 10-30% household bleach, stained in 1% safranin and mounted in glycerin (modified from Cutter, 1978). Ten fields were examined and photographed on each leaf surface. Stomata and leaf epidermal cells were counted from the photographs to obtain the stomatal index ((number of stomata/number of stomata + the number of epidermal cells) x 100/unit area) (Cutter, 1978). Stomatal index appeared to be a more precise method of comparing stomatal numbers between leaves of different sizes than stomatal frequency. Both leaf surfaces were examined for stomata, trichomes, and other epidermal features.

Friedman tests were used to compare tagged, new and total leaf numbers, during successive weeks, within treatments (Kleinbaum and Kupper, 1978). Univariate one-way analysis of variance and Scheffe allowances were used to compare plantlets between soil treatments (Kleinbaum and Kupper, 1978). The level of significance employed was $p \leq 0.10$ for the multiple comparisons, in all other cases $p \leq 0.05$.

Results and Discussion

Leaves of plantlets formed in culture were smaller, thinner and had smaller cells than leaves formed on plants in soil. While absolute cell sizes were smaller than in control plant leaves, the proportions of cell and tissue widths to total leaf widths were similar. Leaves *in vitro* were usually unifoliate, control leaves trifoliate. Plantlet leaves had one layer of palisade cells; wide at the top and narrow towards the base (obconical type or funnel-cells; Drummond, 1965; translation of Haberlandt, 1884). Control plant leaves usually had two or more layers of rectangular palisade cells (Fig. 2a-b). Leaves in culture were less compact compared to controls, allowing more air spaces in mesophyll tissues. Cultures incubated at the higher light intensities had marginally more "normal-shaped" palisade cells than at lower intensities (Fig. 2c-d). Tagged leaves, after 4 weeks in soil, retained most of the features of leaves *in vitro*.

New leaves formed during the first month in soil at the three light intensities used were similar internally but showed a reduced density of parenchymatous tissue compared to greenhouse or field control leaves (Fig. 2e-f). The number of palisade cell divisions appeared to be a function of both leaf age and ambient light intensity as the ratio of palisade cells to epidermal cells increased with age in control leaves and

Figure 2. Photomicrographs of cross sections of red raspberry leaves from field control plants, aseptically cultured plantlets and new leaves of transplants. The bar is 0.05 mm.

A. Young field grown control.

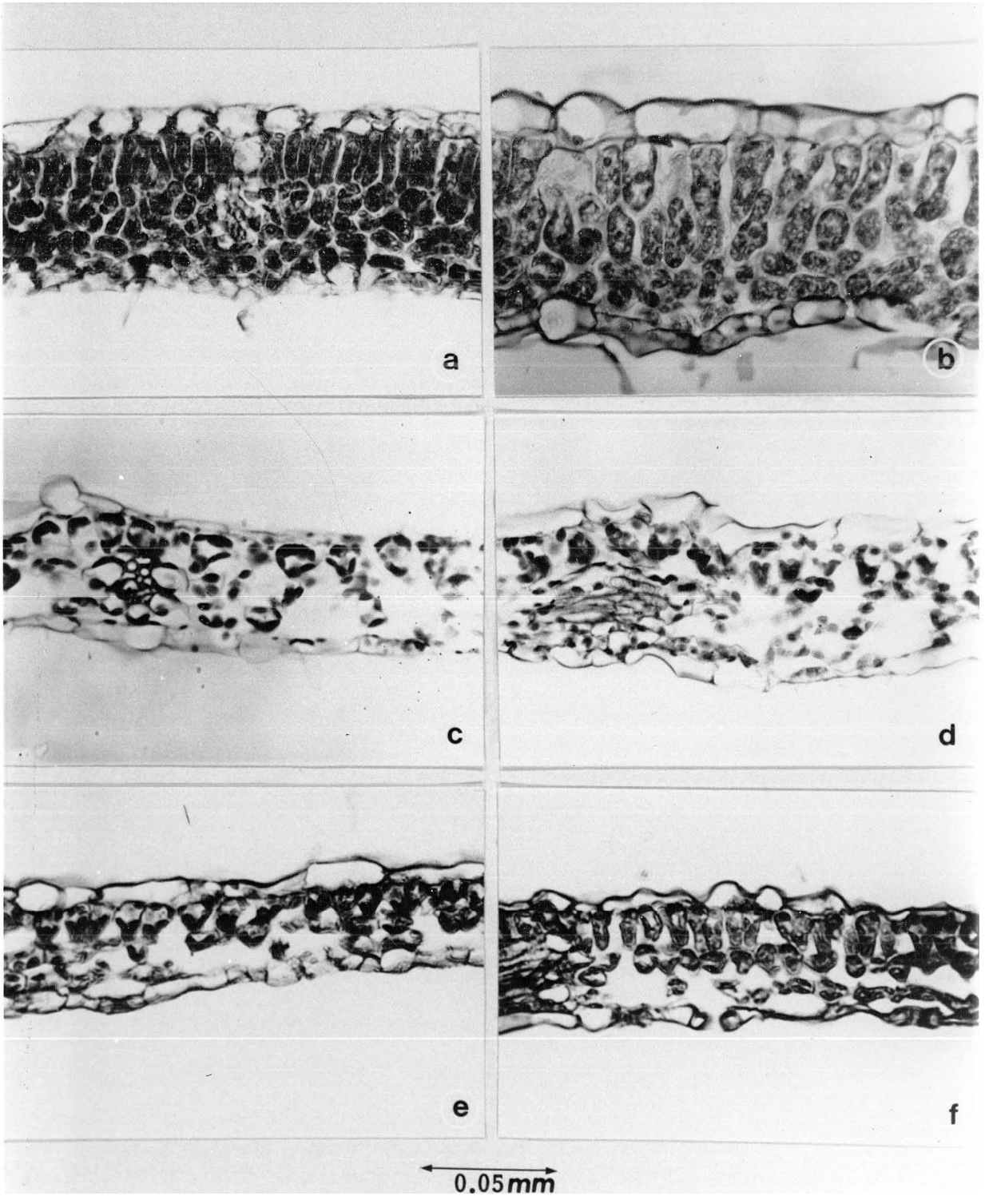
B. Mature field grown control.

C. Plantlet incubated at 2,000 lux.

D. Plantlet incubated at 6,000 lux.

E. Transplant, new leaf at 3,000 lux.

F. Transplant, new leaf at 9,000 lux.


a
b
c
d
e
f
0.05mm

higher light intensity in mature new leaves of transplants. The reduced density of palisade cells found in leaves of plantlets and recent transplants imply lower than control rates of palisade cell division. It is possible that asynchrony in epidermal cell and palisade cell expansion stresses and therefore widens the upper portion of the palisade cells, creating the obconical shape. New leaves that developed after the plantlets had been in soil for 2 months were similar to control leaves.

Calcium oxalate crystals (Metcalfe and Chalk, 1950) were observed in cultured leaves and in greater numbers in control leaves. They were located in palisade cells in cultured leaves. They were present in the upper palisade cells and in the spongy parenchyma adjacent to the lower epidermis in control leaves.

Stomata were anomocytic (Metcalfe and Chalk, 1950). Four to six irregularly shaped cells surrounded each stoma. The stomata were polymorphic, ranging in size from 18-39 um in length and 13-36 um in breadth, and distributed one to many cells apart. The guard cells were kidney-shaped and flush with the epidermal cells in control plant leaves, slightly raised in leaves of plantlets. Epidermal cells were polygonal or irregular in the main veinal areas with straight anticlinal walls and irregularly shaped in between the main veins with undulate anticlinal walls. Leaves of plantlets were amphistomatic with stomata in greater numbers on the lower leaf surfaces. Stomata were present frequently on the leaf peripheries (Fig. 3a). Stomatal index per

mm^2 of cultured plantlet leaves ranged from 5-50 on the adaxial surface and 100-200 on the abaxial leaf surface. Adaxial and abaxial surface stomata were not different from one another in size. Leaves from control plants were predominantly hypostomatic; stomata were scarce on the upper leaf surface, never on the leaf peripheries. Stomata found on the upper surface were clustered near the leaf tip. After plantlets were transferred to soil from culture, new leaves formed during the first month at 3,000 or 6,000 lux, had the external features of cultured leaves; adaxial and peripheral stomata were present and the stomatal index was similar. New leaves from transplants in soil for 2 months at these light intensities had none of these external features and the stomatal index was increased. At the highest light intensity (9,000 lux) all new leaves were hypostomatic, and peripheral stomata were rare. The stomatal index of these leaves was greater than 350 per mm^2, higher than in culture, and similar to controls. Stomatal index of tagged leaves was similar to *in vitro* ones.

Adaxial stomata are not common among the Rosaceae (Metcalfe and Chalk, 1950). Possibly they are induced environmentally as all *in vitro* formed leaves had them, while new leaves initiated in soil from culture-derived plantlets sometimes posessed this feature. Cutter (1978) describes stomatal variability as a humidity related phenomenon although light intensity may be implicated (Gay and Hurd, 1975; cited by Cutter, 1978). In the example cited, of tomato, increased light intensity caused the

normally hypostomatic tomato leaves to become amphistomatic, a situation opposite to one extant in these red raspberry experiments. More must be learned about the control of stomatal distribution and frequency.

Four types of trichomes were found on the leaves of this red raspberry selection. Unicellular, thick-walled, falcate or straight hairs were located primarily on the adaxial surface, especially along the adaxial leaf margins. They were uniform along the leaf margins, but varied in length on the lamina. There were more trichomes along the leaf margins in control leaves than in cultured ones. They alternated in a regular pattern with colleters in culture and on new leaves of transplants. In control leaves colleters rarely were found marginally (Fig. 3a-c). Glandular, multiseriate stalk, multi-celled head colleters (Radford, Dickison, Massey and Bell, 1974) were found primarily on the abaxial leaf surface and margins and the petiole of plantlets and new leaves of transplants. Some were freestanding or forming secretory leaf teeth on the margins. They were taller on the petioles with secondary wall thickenings of the stalk and ephemeral heads (Fig. 3d-f). Setose hairs (multiseriate, erect, straight, stiff trichomes which taper from a stout base; Radford et al., 1974), were located on the petiole and abaxially on the main veins close to the petiole. These were transparent at first then bright red as they matured. They occurred in culture but more abundantly on leaves grown in soil (Fig. 3g). Unicellular, thin

Figure 3. Photomicrographs of cleared and safranin-stained red raspberry leaves showing the margins, the abaxial surfaces of cultured and control leaves and the four different trichome types.

A. Leaf margin of aseptically propagated plantlet; colleters alternate with straight or falcate hairs, peripheral stomata present.

B. Leaf margin of greenhouse or field control plant; colleters absent, straight or falcate hairs entangled with filiform hairs.

C. Unicellular, thick walled, falcate or straight hair.

D. Freestanding marginal colleter showing multiseriate stalk, multi-celled head.

E. Marginal colleter forming secretory leaf tooth.

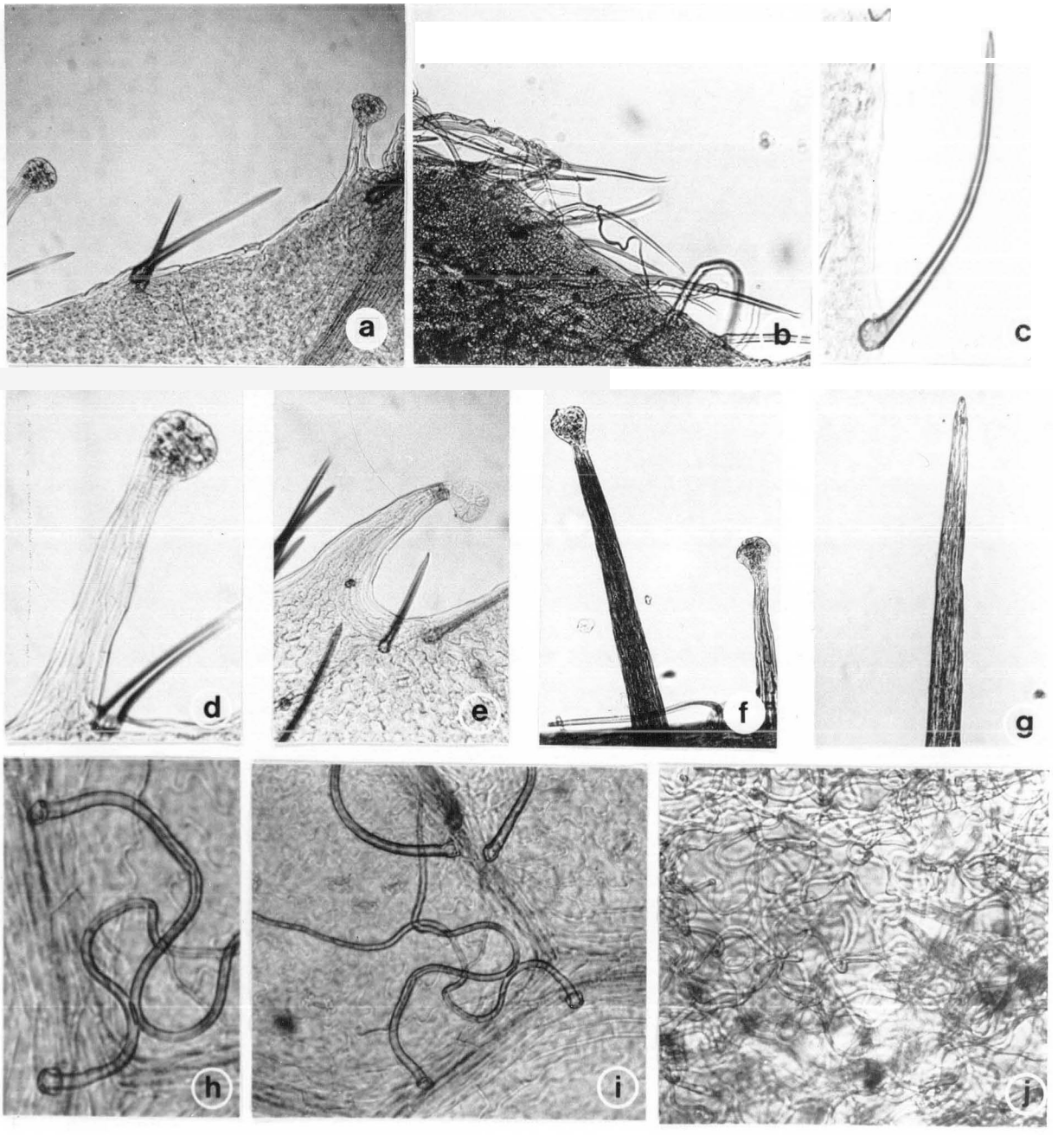
F. Petiolar colleter showing secondary wall thickenings approaching but not including the ephemeral secretory head.

G. Setose trichome; multiseriate, erect, straight, stiff, tapering from a stout base.

H. Filiform trichome.

I. Abaxial leaf surface of aseptically propagated plantlet leaf showing open stomata, sparse filiform trichomes.

J. Abaxial leaf surface of control plant leaf showing mat of entangled filiform trichomes.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walled, flexuous, filiform hairs were located on the abaxial lamina and were numerous on the petiole. They were less on the main veins close to the petiole in low light intensity cultures. Occasionally in high light intensity (6,000 lux) cultures and usually in control leaves they covered and obscured the entire lower leaf surface. They occurred in intermediate numbers in new leaves. These varied in length and in degree of circuitousness (Fig. 3h-j).

The presence of filiform hairs may limit transpiration losses (Drummond, 1965; translation of Haberlandt, 1884) from hypostomatic leaves. Their almost complete absence on leaves formed in culture offers no protection against water loss from the abaxial surface of leaves of plantlets transferred from culture to soil. Furthermore, their absence from the leaf margins and the presence of peripheral stomata on the newly transferred plantlet leaves increases the likelihood of water stress.

Transplant survival rates were 90-95%. There was no difference in transplant survival in soil related to light intensities but transplant size showed an increase with increasing light intensity. Tagged leaves were retained for different lengths of time after the plantlets were transferred to soil. There was a significant decrease in tagged leaf number by the 3rd-4th week at all light intensities. The 4th week after transplant old (tagged) leaves made up only 40-45% of the total leaf number and continued to decline in numbers for up to 3

months following transplantation. Retention was not associated with light intensity. New leaves appeared more rapidly at 9,000 lux, 2 weeks after transplantation, than at 3,000 or 6,000 lux. However, there was no significant difference in total new leaf number at any of these intensities by the end of the 4th week. Leaf area though, appeared to increase with light intensity.

It is apparent that red raspberry plantlets grown in vitro possess anatomical and leaf surface features induced by this environment; these are epigenetic effects. Some of these anatomical features are characteristic of herbaceous plants growing under conditions of ample available moisture such as reduced palisade tissue development, large intercellular spaces, thin leaves, epidermis with thin cuticle and raised stomata (Esau, 1965). These anatomical and leaf surface features all contribute to water stress of new transplants. They vary somewhat between individual leaves of plantlets in culture, and may be reflected in their differential retention time after transplant. The first new leaves of transplants are transitional in their anatomical and leaf surface features. The number of these transitional leaves may depend on the number of immature leaf buds formed in culture. The degree of transition of these leaves and how closely they resemble the cultured or the control plant leaves may be a reflection of the stage of maturity of these leaf buds when the plantlet was transferred to soil and the conflicting stresses imposed by both the culture environment and their new soil environment.

II. The Petiole, Leaf, Stem and Root Anatomy of Red Raspberry in Culture and Transferred to Soil

Introduction

Little is known of how the anatomy of micropropagated plantlets is affected by the culture environment or how anatomy of transplants is modified during acclimatization to the soil environment. Of the reports which describe foliar anatomy and surface features of plantlets and transplants (see General Introduction; Chapter I and Table 1) none explore petiole, root or stem anatomy. Evaluation of structural changes taking place during acclimatization is prerequisite to comprehension of this process and necessary to the development of more efficient transplant protocols (Wetzstein and Sommer, 1983). In previous investigations, plant leaves have been sampled at random 2-6 weeks after transplant, with no knowledge of the stage in the acclimatization process at which these leaves were formed. In this study leaves formed in culture were labeled at transplant and new leaves labeled weekly thereafter so petioles, leaves and subjacent stem tissue could subsequently be identified as to period of development (measured in weeks from the time of transplant). As leaf anatomy of red raspberry plantlets and transplants has been discussed in detail elsewhere (Donnelly and

Vidaver, 1984a; also see Chapter I) only additional findings are mentioned here.

Structural features of the petiole, leaf, stem and root anatomy of red raspberry plantlets grown in aseptic culture, before and after transplantation, were examined. The way in which light intensity affected anatomy of cultured plantlets and the survival and anatomy of transplants and the assessment of persistent organs (leaves, petioles, stem retained from culture) and changes in anatomy of new organs formed during the acclimatization process in soil were of special interest.

Materials and Methods

Plantlets were cultured at low and high light intensity (2,000 and 6,000 lux) and leaves were tagged at the time of transplantation. Twelve rooted plantlets were transferred to soil at each light intensity. Plantlets from 2,000 lux culture were transplanted to 3,000, 6,000 and 9,000 lux in soil. Plantlets from 6,000 lux culture were transplanted to 6,000, 9,000 and 12,000 lux in soil.

At weekly intervals transplants were examined, and any new leaves formed during that week were tagged. After 5 weeks in soil transplants had markers of 6 different colours indicating the period of tissue formation. In this way leaves, petioles and subjacent stem tissue could be evaluated for changes occuring in successive weeks during acclimatization. It was unclear at the time of sampling whether new leaves that had expanded in the

soil environment had developed from leaf buds initiated in culture or in soil. Root samples were collected from transplants after they had been 5 weeks in soil. It was unclear at the time of sampling when they had formed after transplant, or whether they were actually persistent from culture.

Anatomical features were examined from plantlets, persistent and new tissues of transplants and primocane and florocane tissues of 2 year old field control plants. Petiole, (portion subjacent to the leaf), leaf (mid-tip area), stem (subjacent to the petiole) and root samples were cut into small sections, fixed in formalin-acetic acid-alcohol, dehydrated in an ethanol series and wax-embedded. These were sectioned (8-10 um), stained and counterstained in safranin and fast green and mounted in permount (Sass, 1958). Micrographs were taken from the prepared slides and the prints examined. The general shape, size and integrity of each organ was noted and support tissue (sclerenchyma, collenchyma and secondary wall deposition) described.

Results and Discussion

Few plantlets (5/36) from high light intensity (6,000 lux) culture survived transplantation to 6,000, 9,000 or 12,000 lux soil conditions and therefore were not sampled. Of the 36 plantlets transplanted from low light intensity culture (2,000 lux) 33 survived. The 3 mortalities occurred in transplants to

9,000 lux. This and previous unsuccessful attempts to transplant from 2,000 lux culture to 12,000 lux in soil suggest that 9,000 lux is probably the upper limit to which plantlets incubated at 2,000 lux can be transplanted successfully. The pronounced differences in survival rate between transplants from cultures incubated at low and high light intensity may be explained in part by the presence in the lower light intensity cultures of greater pigment content (Donnelly and Vidaver, 1984b; Chapter III), and possibly smaller leaf size which may reduce the amount of water stress (Donnelly and Vidaver, 1984a,b; Chapter I, III) and deformation when desiccation occurs (Wetzstein and Sommer, 1983) following transplant.

Petioles of red raspberry plants have three vascular bundles; one large flattened arc and two circular rib bundles. Petioles of controls were surrounded by four to five cell layers of collenchyma tissue beneath the epidermis and developed sclerenchyma caps six to eight cell layers deep in the phloem tissues of the three leaf traces. Petioles of *in vitro* plantlets had diameters only one quarter to one third that of control plants and lacked collenchyma and phloem sclerenchyma (Fig. 4a,b). The persistent leaf petioles of transplants did not develop collenchyma but those of new leaves, even those formed the first week after transplant had one to several layers of collenchyma cells. The vascular tissues and parenchyma cells had thicker cell walls in the new leaf petioles compared to those in plantlets. The petioles of successively formed new leaves

progressively increased in diameter and had an increased number of vascular elements of both xylem and phloem (Fig. 4c).

Mature leaves of red raspberry control plants were usually trifoliate and had prominent veins. The abaxial surface layers in the veinal areas had very thick-walled collenchyma cells. Leaves in culture and those of transplants were unifoliate (Donnelly and Vidaver, 1984a; see Chapter 1) and lacked collenchyma. The amount of leaf dissection in Ranunculus is apparently related to temperature, with clefts or notches in the primordial leaf margins that cause lobing in the mature leaf, appearing at reduced temperatures (Fisher, 1960). Effects of elevated temperature exposure may persist for several months (Fisher, 1954) and may account for this suppression in the new leaves of red raspberry transplants. Vascular organization in both plantlets and transplants was similar to control leaves. The epidermal cells of cultured leaves were often somewhat collapsed. This has been observed in leaves of sweetgum plantlets (Wetstein and Sommer, 1983), and is likely related to elevated relative humidity (Esau, 1965). Epidermal cell distortion or collapse was frequently noted in new leaves of transplants at all light intensities. The ratio of plantlet palisade cells to epidermal cells was usually 1:1 or 2:1, as it was in very young control leaves. In older control leaves this ratio increased to many:1 as epidermal cell expansion and palisade cell division proceed. The sizes of the leaves of transplants and the palisade:epidermal cell ratio was greatest,

the sizes of the substomatal cavities was smallest, in those transplanted to the highest light intensity (9,000 lux). Size of the substomatal cavities has been correlated to the amount of water stress: large where relative humidity is high (Manning, Miller and Teare, 1977). Wetzstein and Sommer (1983) use the observation of Bunning (1952), that stomata develop in areas where the epidermis is not in contact with the mesophyll, to support their findings of greater stomatal density in cultured sweetgum leaves, where the mesophyll is loosely packed. In red raspberry plantlets stomatal index is lower in vitro despite the relatively loose packing density of the parenchymatous tissues (Donnelly and Vidaver, 1984a; also see Chapter I) as it is in 'Pixy' plum (Brainerd, Fuchigami, Kwiatkowski and Clark, 1981). While the vasculature of the leaves of the 9,000 lux transplants approximated the control leaves the compactness of the parenchymatous tissues was not as great and the shape of the palisade cells was abnormal even in new leaves formed 5 weeks after transplant.

Stems of control red raspberry plants had a continuous cylinder of collenchyma in the cortex beneath the epidermis, a thick layer of sclerenchyma in the phloem of the stele and both thin and thick-walled pith cells (Fig. 4d). Stems of in vitro plantlets were much smaller in diameter than control plant stems. They had little collenchyma in the cortex, very few sclerenchyma cells and no thick walled pith cells (Fig. 4e). The transplants had new stem growth with intermediate amounts of

collenchyma in the cortex, some sclerenchyma cells in the phloem and some thick walled pith cells (Fig. 4f). Transplants at the higher light intensities had greater support tissue development and larger stem diameters. The base of the plant, location of the persistent portion of the stem changed little from the culture type even 5 weeks after transplant. Stem tissue persistent from culture increased only slightly in diameter through cell expansion and wall deposition. This may be a limiting feature in transplant development. It is possible activity of the vascular cambium, in a later stage of development, increases the amount of support tissues. It is also possible that in the case of red raspberry, where new canes are constantly produced from shoot bases and roots, the canes arising from culture derived tissue do not feature prominently in the transplant's future.

Roots of control red raspberry plants were brown in colour and had a multilayered periderm. The roots of cultured plantlets were white or pinkish in colour and covered with fine root hairs. They were much smaller in diameter and had little periderm (Fig. 4g,h). The roots of transplants examined were brown, equally small, and had intermediate periderm development (Fig. 4i). Little is known of the functional capability of the persistent roots, or of those formed after transplant, compared to control roots. The lack of root hairs on many varieties of *in vitro* plantlets grown on solidified medium has led to some question of their functional capability; when observed 2 weeks

after transplant, the *in vitro* formed roots had apparently died, and new roots started to develop (Debergh and Maene, 1981). The small, delicate roots of cultured plantlets and plants 5 weeks after transplant suggest some differences in their physiology compared to control roots. This is supported by evidence of Poole and Conover (1983) that roots of *Dieffenbachia maculata* (Lodd.) transplants were not yet acclimatized 6 weeks after transplant to soil. These differences may limit transplant development until control type roots are produced by the transplants at some later stage. If the situation with roots is analagous to that of shoot growth, and there is some evidence for this in the intermediate type of roots observed 5 weeks after transplant, then transitional root types may develop during acclimatization of transplants.

In leaves, stems and petioles the amount of support tissue and wall deposition was greatest in the higher light intensity transplants. This was apparent from greater ease of sectioning of the firmer tissue and greater staining intensity and thickness of some cell walls such as the vessel elements of the xylem. This is supported by data showing significantly more dry weight accumulation in leaves at the higher transplant light intensities (Donnelly and Vidaver, 1984b; see Chapter III). Despite the progressively younger age of the tissue tested, the compactness of the leaf tissues, amount of wall deposition and size of transplant organs, at the three light intensities, showed a distinct increase that was inversely related to time of

organ appearance after transplant.

Scarcity of collenchyma tissue in culture and reduced amount in the new leaves and petioles of transplants compared to controls may be explained in part by results of Razdorskii (1955) and those of Venning (1949) cited by Esau (1965). They found that in developing plants exposed to mechanical stresses, collenchyma wall thickenings began earlier and became more massive than in plants unexposed to such stresses. In culture it would seem that mechanical stresses are few; there is little or no air movement in stationary cultures. Even the first new leaves of transplants develop some collenchyma tissues, the later formed leaves have much more. The implication here is that control levels of collenchyma cell wall development cannot be expected in culture and also may be inhibited by the intermediate environment into which the transplants are placed, as both are relatively tranquil in terms of mechanical stress.

Scarcity of sclerenchyma cells and secondary wall development is probably related to the elevated relative humidity of both the culture environment and the intermediate transplant environment. Eberhardt (1903; cited by Wangermann, 1961) found reduced sclerenchyma and secondary wall formation (including lignification) in both roots and stems of a wide number of species subject to conditions of high relative humidity.

Persistence of *in vitro* features in organs appearing after transplant may be due to one or a combination of the following

events:

a) Organs formed after transplant had been initiated in culture, and defined in the early stages of development (as primordia) by the culture environment.

Organs formed after transplant and initiated in soil, are prevented from rapidly achieving the control type structure because they are limited in their development by:

b) culture environment effects which can only gradually be escaped from,

c) the ambient intermediate type environment which enables survival through the development of transitional organs,

d) the retention of culture type organs that make up the existing transplant structure and influence the physiological status of the rest of the plant.

Support for these suggestions is merely circumstantial; evidence for (a) is found in occasional organs (leaves or petioles) that did not follow the sequence or cline towards the control type anatomy, apparent in successive weeks growth after transplant. In these cases leaf buds may have been quiescent for several weeks, only expanding after transplant. Alternatively, it may be important where on the shoot the leaves emerge from, so leaves expanding in any one week at the shoot apex or the axils of basal leaves may look quite different. Experimental studies by Ashby (1948) and Ashby and Wangermann (1950) lend support to explanations (c) and (d); the interplay of developing parts in relation to structural gradients (undetermined influences of

immature leaves on still younger leaves, and processes of aging in the apical meristem) and microclimate. In 1961, Wangermann suggested that these undetermined influences, involving water supply and humidity dependent developmental effects, related to the balance and distribution of growth hormones. Some evidence for (b) and (c) is found in the results of experiments done with ferns. A progressive increase in leaf size and complexity (heteroblastic development) has been attributed to changes in the nutritional status of the plant, especially the carbohydrate level. Goebel (1908; cited by Steeves and Sussex, 1972) suggested that small and morphologically simple leaves of juvenile stage ferns reflected their restricted nutritional status. Increased size (Steeves, 1963) and morphological complexity (Sussex and Clutter, 1960) of later formed leaves was shown to be the result of increased cell division and attributed to enhanced carbohydrate nutrition. Steeves and Sussex (1972) summarize the difficulty in interpreting control mechanisms involved in leaf form: "It is not possible to determine to what extent the factors that influence leaf development act directly upon the leaf and to what extent they operate through the parent plant".

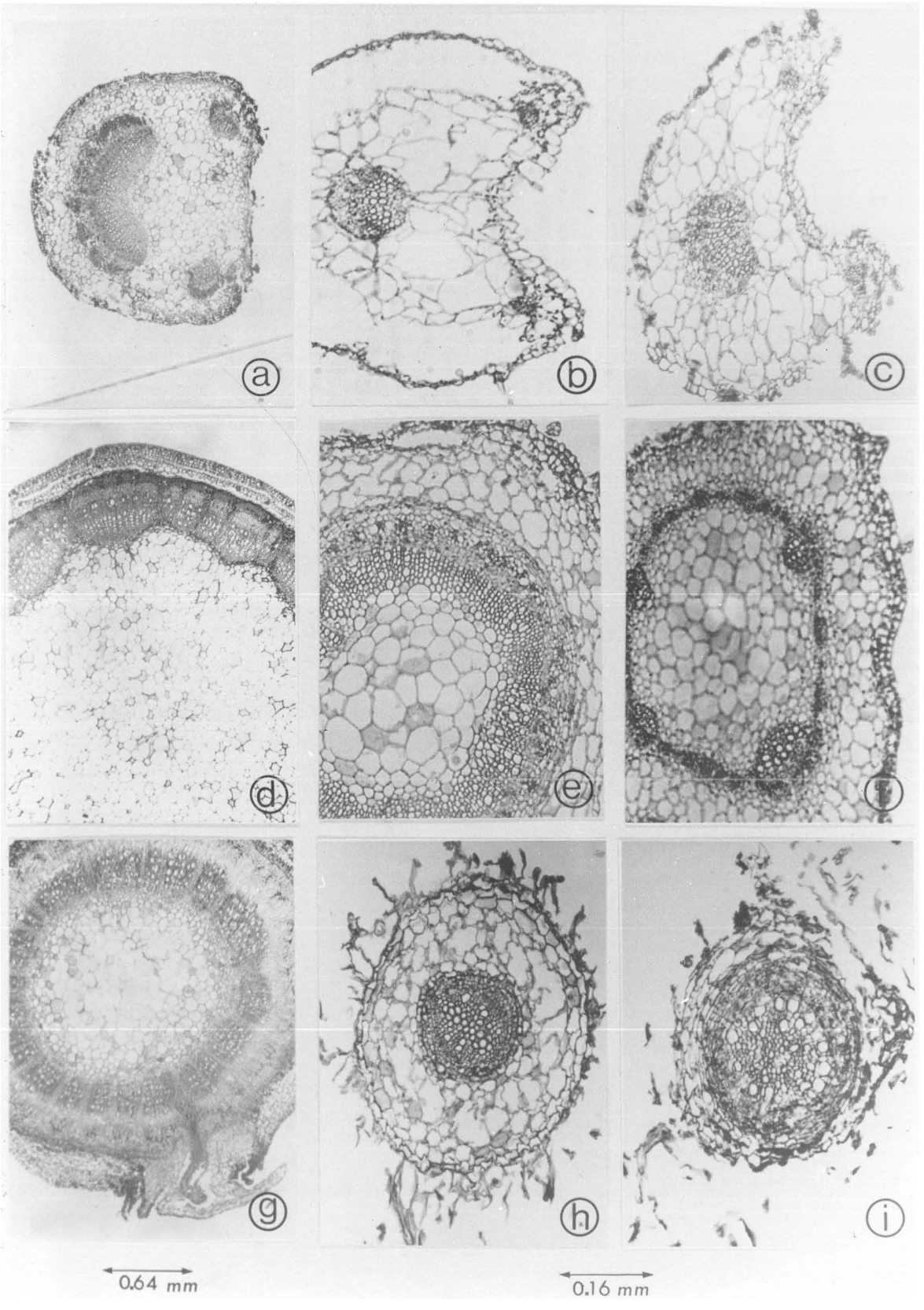
Whatever the underlying reason(s) a graduated return to control type development, during acclimatization to the soil environment, accelerated by higher light intensity is indicated. Only organs arising after transplant can approach control type development. Thus the process referred to as acclimatization

must be considered as leading to the culmination of linear, irreversible tissue or organ development. Persistent leaves, petioles and stem tisues clearly do not redifferentiate to a form suited to the changed environment as was implied by Grout and Aston (1978). Redifferentiation does not occur in persistent red raspberry tissues; the organs grow minimally, some secondary wall deposition occurs which may account for the small increases seen in the dry weights (Donnelly and Vidaver, 1984b; see Chapter III), at the higher transplant light intensities. Anatomical variations observed in cultured red raspberry plantlets result from stresses imposed on developmental regulation. The culture environment is not so far from the normal for this genotype that development is completely inhibited (as it is for the many species, many of them xerophytes, that are incapable of surviving in vitro). The process of acclimatization involves reversion to control type developmental regulation; this takes time, proceeds in a stepwise manner and is apparently a highly complex activity whose mechanisms remain to be elucidated.

In Table 1 the epigenetic changes seen in leaves, petioles, stem and root anatomy of tissue cultured plants propagated by axillary bud proliferation are compiled from the literature and studies of red raspberry including Donnelly and Vidaver (1984a; also see Chapter I-II). This is intended to be a complete account of all such variation observed, that can be attributed exclusively to environmentally induced rather than genetic

change. An attempt has been made to correlate epigenetic variation with the major environmental stresses in culture from published accounts of environmental effects on plant morphology. The immense literature concerning environmental or climatalogical effects on plant morphology, physiology and reproductive behavior was reviewed in the volume edited by Evans (1963), and by Bieleski, Ferguson and Cresswell (1974). Additional sources utilized, include: Baker, 1974 and Darrell and Ferree, 1983 (environmental effects on plant cuticles, waxes); Corre, 1983 (effects of light on plant development); Esau, 1965 (environmental effects on plant morphology); Gay and Hurd, 1975 (environmental effects on stomata); and Manning, Miller and Teare, 1977 (effects of relative humidity on leaf morphology). This table is intended to serve as a preliminary framework for future investigation of *in vitro* induced variation. Better understanding of the complex interactions between environment and development during the *in vitro* and the transitional acclimatization stages of tissue culture propagation is essential to the application of the most effective methods for producing viable, self-sustaining plants.

Figure 4. Photomicrographs of sectioned petioles (A-C), stems (D-F) and roots (G-I) of control plants (left), plantlets (centre) and transplants (right). The bar is 0.64 mm (controls), or 0.16 mm (plantlets and transplants).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0.64 mm
0.16 mm

Table 1. Epigenetic changes in anatomy of plantlets induced by the culture environment. The * indicates this chapter.

Anatomical variation	Plant	Environmental determinants
Foliage		
shape change:	R (D+V, 1984a, *)	T (F, 1954, 1960)
size change:		
very thin	R (D+V, 1984a)	RH L (M,M+T, 1977; C, 1983)
	S (W+S, 1982)	RH, T (F, 1960)
	C (S+L, 1979)	
very small	R (D+V, 1984a)	RH (M,M+T, 1977)
cell size decrease:		
all	R (D+V, 1984a)	
adaxial epidermal	S (W+S, 1982)	
palisade length	P (B,F,K+C, 1981)	
cell type change:		
decreased:		
collenchyma	R (D+V, 1984*)	MS (R, 1955; V, 1949)
sclerenchyma	ibid	RH (Eb, 1903)
cell wall thickenings	ibid	RH
cuticle		
undeveloped	S (W+S, 1982)	RH, L (intensity and quality) nutritional status, physiological function (M+J, 1970).
epicuticular		
wax amount:		WP (D+F, 1983)
less	S (W+S, 1982)	RH, L, T (B, 1974)
	CC (W,Q+S, 1979)	RH (Z,M+H, 1983)
	P (F,C+S, 1981)	
	CH (W,D+S, 1983)	
	C (Z,M+H, 1983)	
& structure:		
not changed	S (W+S, 1983)	
changed	CA (G+A, 1977a)	
	CC (G, 1975)	
	C (S+L, 1979)	
stomatal		
density:		L (G+H, 1975)
increased	S (W+S, 1983)	RH (M,M+T, 1977)
frequency:		
decreased	P (B,F,K+C, 1981)	
index:		
decreased	R (D+V, 1984a)	
distribution:		
altered	R (D+V, 1984a)	RH, T, L (G+H, 1975)

size:			
increased	S	(W+S, 1983)	
no change	R	(D+V, 1984a)	
aperature:			
open	R	(D+V, 1984a,*)	RH, L
	CH	(W,D+S, 1983)	
	AA	(B+F, 1981, 1982)	
	P	(B+F, 1981)	
	CA	(W, Q+S,1979)	
		(W, D+S,1983)	
	S	(W+S, 1983)	
closed	CA	(G+A, 1977a)	
shape:			
changed	S	(W+S, 1983)	
substomatal cavity:			
increased	R	(D+V, 1984*)	RH (M,M+T, 1977)
guard cells:			
protruding	S	(W+S, 1982,1983)	RH (E, 1960)
	R	(D+V, 1984a)	
trichomes:			
absent	AC	(L, 1978)	L,T,RH (F, 1960)
frequency:			
decreased			
& distribution:			
altered	R	(D+V, 1984a)	
epidermal cell:			
distortion	R	(D+V, 1984*)	
	S	(W+S, 1982,1983)	
contents			
altered	S	(W+S, 1982)	
ratio to palisade			
cells decreased	R	(D+V, 1984*)	L, RH, mesophyll (E, 1960)
parenchyma tissue:			
limited or absent			RH (E, 1960)
palisade layer	CA	(G+A, 1978)	
	S	(W+S, 1982)	
decreased packing			
density	S	(W+S, 1982)	
	R	(D+V, 1984a)	
increased % air			
in mesophyll	S	(W+S, 1982)	
	P	(B,F,K+C, 1981)	
palisade cell			
shape changed	R	(D+V, 1984a)	
(obconical shape)			
chloroplast			
altered shape			
& ultrastructure:	S	(W+S, 1982)	
irregular membrane system			
not organized into grana			

and stroma lamella				
starch content:				
increased	S	(W,Q+S, 1979)		
decreased	S	(W+S, 1982)		
	R	(D+V, 1984*)		
mesophyll cell				
contents changed:				
ergastic vacuolar				
components	S	(W+S, 1982)		
organelle frequency		ibid		
parietal cytoplasm		ibid		
prominent vacuole		ibid		
Stem				
decrease in:				
stem diameter	R	(D+V, 1984*)	RH	(M,M,+T, 1977)
collenchyma		ibid	MS	(R, 1955; V, 1949)
sclerenchyma		ibid	RH	(Eb, 1903)
wall thickenings		ibid	RH	(Eb, 1903)
periderm		ibid		
thick walled pith cells		ibid		
Petiole				
decrease in:				
petiole diameter	R	(D+V, 1984*)		
collenchyma		ibid	MS	(R, 1955; V, 1949)
sclerenchyma		ibid	RH	(Eb, 1903)
wall thickenings		ibid	RH	(Eb, 1903)
Root				
decrease in:				
root diameter	R	(D+V, 1984*)		
collenchyma		ibid	MS	(R, 1955; V, 1949)
sclerenchyma		ibid	RH	(Eb, 1903)
wall thickenings		ibid	RH	(Eb, 1903)
periderm		ibid		

Key to the Plants:

AA	apple	Malus domestica (Borkh) cv. 'Mac 9'
AC	apple	cv. 'McIntosh'
C	carnation	Dianthus caryophyllus L.
CA	cauliflower	Brassica oleracea var. botrytis cv. 'Armado Tardo'
CC	cauliflower	cv. 'Currawong'
CH	chrysanthemum	Chrysanthemum x Morifolium cv. 'Snowdon'
P	plum	Prunus insititia L. cv. 'Pixy'
R	red raspberry	Rubus idaeus L. BC72-1-7
S	sweetgum	Liquidambar styraciflua

Key to the Environmental Parameters:

C	carbohydrate
CO2	carbon dioxide
L	light
MS	mechanical stress
O	osmolarity
O2	oxygen
RH	relative humidity
T	temperature
WP	water potential

Key to the Authors:

B	Baker
B+F	Brainerd and Fuchigami
B,F,K+C	Brainerd, Fuchigami, Kwiatkowski and Clark
C	Corre
D+F	Darnell and Ferree
D+V	Donnelly and Vidaver
Eb	Eberhardt
E	Esau
F	Fisher
G	Grout
G+A	Grout and Aston
G+H	Gay and Hurd
L	Lane
M,M+T	Manning, Miller and Teare
M+J	Martin and Juniper
M+C	Metcalfe and Chalk
R	Razdorskii
S+L	Sutter and Langhans
V	Venning
W,Q+S	Wardle, Quinlan and Simpkins
W,D+S	Wardle, Dobbs and Short
W+S	Wetzstein and Sommer
Z,M+H	Ziv, Meir and Halevy

III. Pigment Content and Gas Exchange of Red Raspberry In Culture and Transferred to Soil

Introduction

Grout and Aston (1977b) suggested that a major factor in the vulnerability of cauliflower transplants to stress was poor development of the photosynthetic system *in vitro*. The photosynthetic capacity of rosaceous plants propagated by axillary bud proliferation has not been elucidated previously, either in culture, or after transfer to soil, yet these are amongst the most important commercially of all tissue cultured plants. The initial purpose of this study was to evaluate the potential for CO_2 uptake and to determine the photosynthetic pigment content of red raspberry plantlets grown in aseptic culture at transplant time and after one month in soil under controlled environmental conditions. The way in which light intensity affected photosynthetic performance and pigment content and an assessment of the contribution to the plant both by the original leaves retained from culture and by those formed subsequently in the soil environment were of special interest. A variety of leaf parameters hitherto unreported for cultured plantlet leaves and those of new transplants, including leaf fresh/dry weight ratios (FW/DW), percentage water content (%

WC), dry weight/area (g DW/dm²) and plantlet or transplant total leaf area (TLA) were also evaluated. These parameters were contrasted, where possible, to those of field grown control plants.

Materials and Methods

Plantlets were incubated at 5 light intensities, from 2,000 to 6,000 lux. Gas exchange, pigment content, FW/DW, % WC, g DW/dm² and TLA/plantlet were evaluated at the end of each 4 week culture cycle, the time at which these rooted plantlets were either subcultured to fresh medium or transferred to soil.

Leaves of plantlets from 3,000 lux culture were tagged by tying colored thread around the base of each petiole. Eighteen plantlets with leaves tagged in this manner were transferred to soil at each of three light intensities, 3,000, 6,000 and 9,000 lux. After 4 weeks, 6 transplants from each treatment were used for gas exchange experiments. The remaining plants were utilized for the remainder of the tests.

Carbon dioxide uptake was determined for plantlets, transplants and control plant leaves using a Beckman infrared gas analyzer (IRGA). The temperature in the sample chamber was held at 25 °C (Tele-thermometer reading) by circulating tap water through its water jacket. The light source was three cool white fluorescent bulbs. The intensity was measured with a Li-cor photometer and controlled by adjusting the light source

distance from the sample chamber. Intensities employed to test plantlets and control plant leaves ranged from 4,000 to 24,000 lux, and included the range from very low to saturated uptake rates. The sample was exposed to the lowest light intensity first, then the light was stepped up at 4,000 lux intervals. Two or three readings were taken at each light intensity. Gas exchange was allowed to proceed at saturating light intensity (20,000 or 24,000 lux) until the CO2 compensation point (CP) was reached, after which a dark respiration rate (DR) was obtained. Transplants tested after 4 weeks in soil were evaluated at 20,000 lux and 5-6 readings were taken. They were tested a first time as intact plants and a second time with all tagged leaves clipped off at the base of the petiole. The petioles were clipped off when the sample was removed from the IRGA and the leaf area was measured. Both fresh weight and dry weight (minimum 48 hours in a 50 °C oven) were determined. The leaf area and time for CO2 uptake through the ambient range (365-265 ul/L) at each light intensity were used to compute the rate of CO2 uptake in mg $CO2/dm^2$/hour (0.1g $CO2/m^2$/hour). Kruskal-Wallis or Mann-Whitney U tests were employed to determine whether or not CO2 uptake rates, DR rates and CP were statistically different at the $p \leq 0.05$ level (Kleinbaum and Kupper, 1978).

Plantlet leaf pigment contents were based on the mean of five determinations for each of three to six culture cycles at each light intensity. Pigment content of new and tagged leaves of transplants were based on five determinations of composite

samples from twelve transplants at each light intensity. Pigment analysis was done on pooled leaf tissue samples of 50 mg extracted in 12.5-25 ml of 100% cold acetone by the method of Sestak (1971). Chlorophyll a, b and total carotenoids were estimated by the constants of Holm (1954). Calculations were made on a dry weight (DW) and a fresh weight (FW) basis. Univariate one-way analysis of variance and Scheffe allowances were used to determine if the means were statistically different; at the $p \leq 0.10$ level for the multiple comparisons, otherwise at the $p \leq 0.05$ level (Kleinbaum and Kupper, 1978).

Leaf discs of known area, fresh and oven-dried, were used to obtain measurements of FW/DW, % WC and g DW/dm^2. Measurements of TLA/plantlet were done as for the IRGA samples. Leaf parameters were compared between treatments using Kruskal-Wallis or Mann-Whitney U tests. The level of significance employed was $p \leq 0.05$.

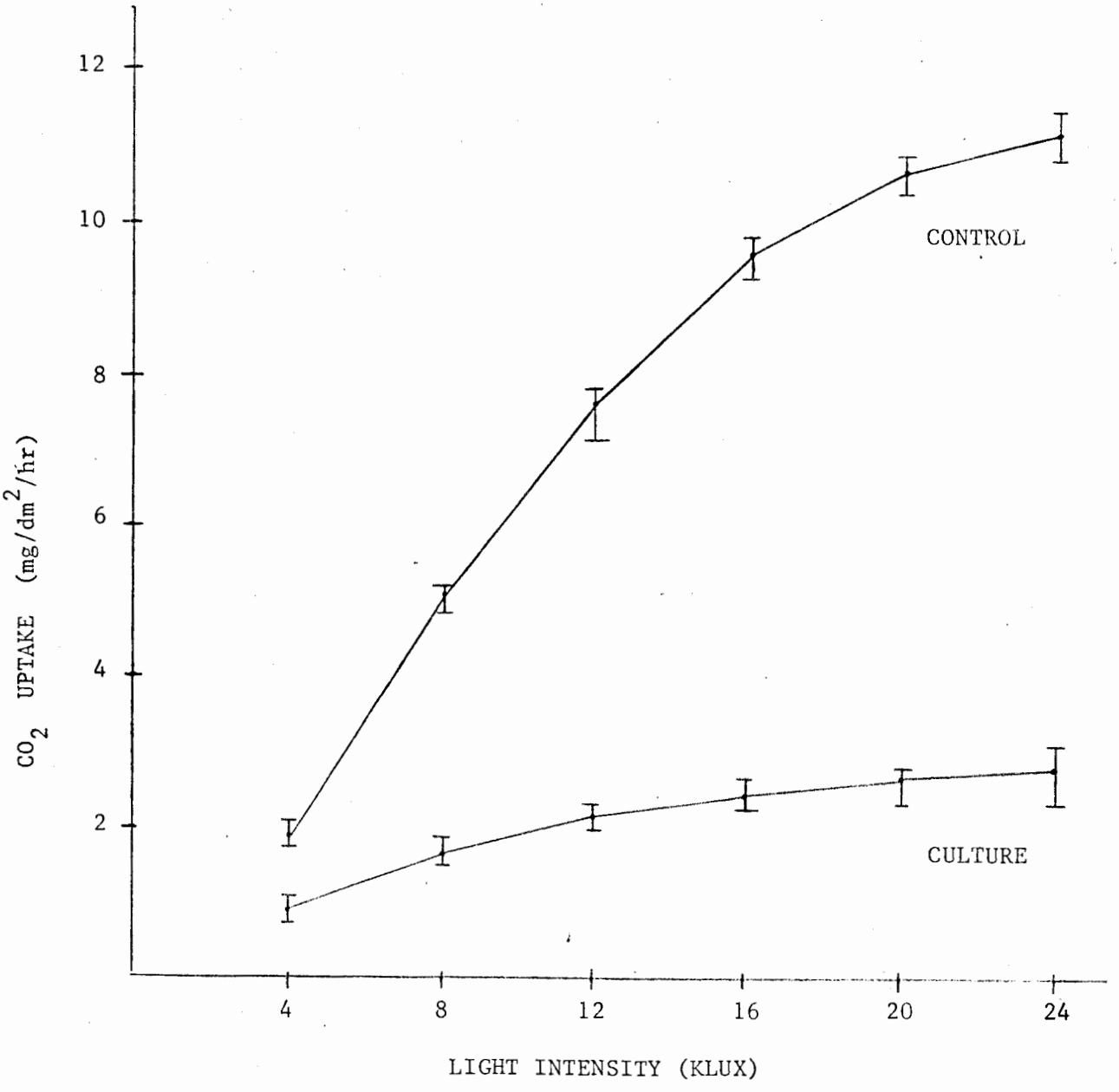
Results

The net CO2 uptake rates of plantlets, examined at the end of a 4 week culture cycle, were not different among culture treatments when compared at a range of light intensities from very low to saturating but were significantly lower than those of field control plants. Carbon dioxide uptake rates of plantlets ranged from less than 1 mg CO2/dm^2/hour at culturing

intensities to 2-3 mg CO_2/dm^2/hour at saturating intensities and rarely exceeded 4 mg CO_2/dm^2/hour. Mature leaves, detached from field control plants during midsummer had maximum uptake rates of 10-15 mg CO_2/dm^2/hour at saturating intensities (Fig. 5).

The CO_2 uptake rates at 20,000 lux for intact transplants averaged from 4.5 to 6.0 mg CO_2/dm^2/hour and were significantly greater than those for plantlets taken directly from culture but did not vary with light intensity (3,000 to 9,000 lux) maintained during 4 weeks for transplants in soil. Transplants with tagged leaves removed also had significantly greater uptake rates than plantlets. These uptake rates averaged from 5.5 to 6.5 mg CO_2/dm^2/hour. When CO_2 uptake rates were compared between intact transplants and those with new leaves only (tagged leaves removed), there was no difference at 3,000 lux, but transplants with new leaves only on them at both 6,000 and 9,000 lux had a significantly higher rate than that of intact plants (Table 2). What then is the contribution of the tagged leaves to the overall carbon balance of these transplants after 1 month in soil? Tagged leaves accounted for 30% of the total leaf area at 3,000 lux yet their contribution was less than 10% of total CO_2 uptake. At 6,000 or 9,000 lux tagged leaves composed 10-30% of the total leaf area and were net respirers. New leaves did not photosynthesize optimally and compared to control plants operated at half the "normal" uptake rates.

Figure 5. The CO2 uptake of red raspberry plantlets in culture and that of detached mature control plant leaves, at light intensities from 4,000 to 24,000 lux.



Dark respiration rates (Table 3) of plantlets cultured at 6,000 lux were not different from control plant rates. However, they were significantly lower than those for plantlets exposed to 2,000-5,000 lux. Compensation points (Table 3) did not differ according to light intensity among cultured plantlets but were significantly higher than those of control plants. This implies that plantlets in culture have a higher photorespiration rate than control plants. The DR rates and CPs of intact transplants or those with only new leaves (tagged leaves removed) were significantly different from those of cultured plantlets, but did not vary with light intensity in soil, and were not different from controls.

Plantlet leaf pigment content on a dry weight basis (Table 4) was significantly greater at 2,000-4,000 lux than it was at 5,000-6,000 lux for chlorophyll a and total chlorophyll and at 2,000-3,000 lux than at 4,000-6,000 lux for chlorophyll b and carotenoids. Plantlets at 3,000 lux had significantly greater leaf pigment content than those incubated under any other light intensity, calculated on a fresh weight basis. Molar ratios of chlorophyll a/b and (a+b)/carotenoids were not affected by light intensity in culture.

On a dry weight basis, chlorophyll a and total chlorophyll were significantly greater in new leaves of transplants than in leaves of plantlets in culture, and did not differ among transplants at the three light intensities (Table 4). Chlorophyll b and carotenoid contents in new leaves of

Table 2. Net CO2 uptake measured at 20,000 lux, for red raspberry plantlets, transplants (intact, new and tagged leaves) and field controls.

Light intensity (lux)	Carbon dioxide uptake (mg CO2/dm²/hour)		
Culture Soil		Transplants with only	
	Intact plant	New leaves	Tagged leaves
Plantlets 3,000	2.57±0.20		
Transplants 3,000	5.92±0.20	6.38±0.20	0.57±0.18
6,000	4.65±0.37	5.70±0.17	-0.21±0.44
9,000	4.59±0.16	6.08±0.25	-0.53±0.17
Field controls	10.53±0.25		

Table 3. Dark respiration rate and compensation point of red raspberry plantlets, intact transplants and field controls.

Light intensity (lux)	Dark respiration rate (mg CO_2/dm^2/hour)	Compensation point (parts per million)
Culture Soil		
Plantlets 2,000	-1.24±0.26	148.20± 5.08
3,000	-1.06±0.10	108.48±13.96
4,000	-1.32±0.08	97.95±12.48
5,000	-1.51±0.20	132.52±22.77
6,000	-2.08±0.36	120.17± 9.72
Transplants 3,000	-1.65±0.17	60.99± 1.19
6,000	-1.90±0.46	63.00± 5.00
9,000	-2.17±0.14	67.65± 3.61
Field controls	-2.40±0.22	57.34± 2.57

Table 4. Chlorophyll (chl) a, b, a + b, carotenoids (car) and molar ratio of chl a/b and (a+b)/car calculated on a dry weight (DW) or fresh weight (FW) basis of red raspberry plantlets, transplants (new and tagged leaves) and field controls (new and tagged leaves).

Light intensity (lux)	Leaves		Pigment content (mg/g)		
			Chl a	Chl b	Chl a+b
Culture	Soil				
Plantlets					
2,000	all	DW	9.17±0.52	3.19±0.16	12.36±0.67
		FW	1.36±0.06	0.48±0.24	1.84±0.10
3,000	all	DW	10.37±0.58	3.61±0.21	13.98±0.78
		FW	1.80±0.07	0.62±0.24	2.42±0.09
4,000	all	DW	8.32±0.51	2.88±0.15	11.19±0.65
		FW	1.48±0.07	0.51±0.13	1.99±0.10
5,000	all	DW	6.99±0.46	2.24±0.13	9.23±0.59
		FW	1.54±0.10	0.49±0.03	2.03±0.13
6,000	all	DW	7.79±0.45	2.67±0.15	10.46±0.59
		FW	1.43±0.10	0.49±0.03	1.92±0.13
Transplants					
3,000	new	DW	12.71±0.70	3.72±0.20	16.43±0.90
		FW	2.39±0.13	0.70±0.04	3.09±0.17
	tagged	DW	12.75±1.35	4.25±0.51	16.70±1.85
		FW	2.14±0.23	0.71±0.09	2.81±0.31
6,000	new	DW	13.55±0.37	3.97±0.11	17.51±0.48
		FW	2.65±0.07	0.78±0.02	3.42±0.09
	tagged	DW	9.38±0.52	3.07±0.15	12.44±0.66
		FW	2.16±0.12	0.71±0.04	2.87±0.15
9,000	new	DW	11.61±0.62	3.47±0.17	15.09±0.78
		FW	2.68±0.14	0.80±0.04	3.48±0.18
	tagged	DW	8.77±0.52	2.98±0.22	11.75±0.70
		FW	2.12±0.13	0.72±0.05	2.84±0.17
Field controls					
young	all	DW	11.22±0.56	3.42±0.11	14.64±0.65
		FW	2.38±0.12	0.72±0.02	3.10±0.14
mature	all	DW	5.40±0.40	1.50±0.12	6.91±0.05
		FW	1.79±0.13	0.50±0.04	2.29±0.17

Table 4. (continued)

Light intensity (lux)	Leaves		Car	Molar ratio: Chl a/b	Molar ratio: Chl a+b/car
Culture	Soil				
Plantlets					
2,000	all	DW	4.03±0.23	2.92±0.07	1.85±0.03
		FW	0.60±0.03		
3,000	all	DW	4.46±0.27	2.95±0.07	1.89±0.03
		FW	0.77±0.03		
4,000	all	DW	3.68±0.17	2.94±0.06	1.80±0.03
		FW	0.66±0.03		
5,000	all	DW	2.97±0.16	3.16±0.07	1.84±0.03
		FW	0.65±0.03		
6,000	all	DW	3.49±0.75	2.97±0.08	1.79±0.03
		FW	0.64±0.04		
Transplants					
3,000	new	DW	4.48±0.26	3.48±0.02	2.21±0.05
		FW	0.84±0.05		
	tagged	DW	4.60±0.46	3.06±0.04	2.21±0.02
		FW	0.77±0.08		
6,000	new	DW	4.51±0.16	3.48±0.04	2.33±0.04
		FW	0.88±0.03		
	tagged	DW	3.50±0.12	3.11±0.04	2.13±0.06
		FW	0.81±0.03		
9,000	new	DW	4.06±0.13	3.40±0.03	2.23±0.05
		FW	0.94±0.03		
	tagged	DW	3.36±0.19	3.00±0.12	2.10±0.05
		FW	0.81±0.05		
Field controls					
young	all	DW	3.91±0.17	3.34±0.12	2.25±0.05
		FW	0.83±0.04		
mature	all	DW	2.51±0.14	3.70±0.07	1.65±0.02
		FW	0.84±0.05		

transplants and in leaves of plantlets were not different. All leaf pigment contents on a fresh weight basis, were significantly greater in cultured plantlets than in new leaves of transplants and did not differ among transplants at the three light intensities. All pigment contents were lower in new leaves of transplants at 9,000 lux than at 3,000 or 6,000 lux on an area basis. The molar ratios of chlorophyll a/b and a+b/carotenoids were greater in transplants than plantlets but did not differ among transplants at the three light intensities.

Chlorophyll a and total chlorophyll of tagged leaves at 9,000 lux were significantly lower than plantlets or tagged leaves at 3,000 or 6,000 lux when pigment contents of tagged leaves of transplants were compared to leaves of plantlets in culture on a dry weight basis. Chlorophyll b and carotenoids in tagged leaves and plantlets were not different. All tagged leaf pigment contents among transplants were significantly lower at 9,000 lux than at 3,000 or 6,000 lux, calculated on a dry weight basis. On a fresh weight basis, chlorophyll a and total chlorophyll were significantly greater in tagged leaves than plantlets, while chlorophyll b and carotenoids were not. Pigment content did not vary with light intensity of transplant in these tagged leaves, calculated on a fresh weight basis. The molar ratio of chlorophyll a/b did not differ between plantlets and tagged leaves, nor among transplants at the three light intensities. The molar ratio of (a+b)/carotenoids was significantly greater in tagged leaves than plantlets, but did

not differ among transplants at the three light intensities. There was no significant difference found at 3,000 or at 6,000 lux between pigment contents of new and tagged leaves of transplants. However, all pigment contents were significantly greater in the new leaves at 9,000 lux, as was the molar ratio of chlorophyll a+b/carotenoids. The molar ratio of chlorophyll a/b was significantly greater in the new leaves at all three transplant light intensities. Pigment content of new leaves at 9,000 lux was not different from that of young control leaves. Neither were the molar ratios of chlorophyll a/b nor (a+b)/carotenoids. There was a net decrease in pigment content with leaf maturity in field control plants, calculated on either a dry or a fresh weight basis.

Fresh/dry weight ratio, % WC, and TLA/plantlet (Table 5) did not differ with light intensity in culture and were significantly lower in control leaves. Gram dry weight/dm^2 did not differ with light intensity in culture but was significantly greater in control leaves (Table 5). Plantlets in culture and intact transplants at 3,000 lux were found to be significantly different from intact transplants at 6,000 and 9,000 lux. New leaves remained significantly smaller in area when transplants were kept at 3,000 lux than when they were transplanted to higher light intensities but light intensity did not affect FW/DW nor % WC of new leaves.

Table 5. Fresh/dry weight ratio (FW/DW), percentage water content (% WC), g dry weight/unit area (g DW/dm²) of leaves and total leaf area/plantlet (TLA) of red raspberry plantlets, transplants (new and tagged leaves) and field controls.

Light intensity (lux)	Leaves	FW/DW	% WC	g DW/dm²	TLA (dm²)
Culture Soil					
Plantlets					
2,000	all	6.61±0.65	84.53±1.62	0.14±0.007	0.120±0.008
3,000	all	7.60±0.42	86.72±0.73	0.14±0.005	0.196±0.014
4,000	all	7.79±0.53	86.98±0.88	0.15±0.011	0.215±0.018
5,000	all	6.61±0.82	84.09±1.57	0.17±0.014	0.175±0.034
6,000	all	7.19±0.43	85.99±0.89	0.16±0.009	0.139±0.009
Transplants					
3,000	new	5.31±0.21	81.12±0.72	0.14±0.005	0.297±0.064
	tagged	5.95±0.91	82.49±2.34	0.15±0.002	0.124±0.024
6,000	new	5.12±0.11	80.44±0.40	0.15±0.005	0.624±0.054
	tagged	5.26±0.48	80.64±1.88	0.15±0.011	0.138±0.051
9,000	new	4.34±0.37	76.64±1.83	0.21±0.021	0.600±0.118
	tagged	4.14±0.67	74.67±3.58	0.20±0.009	0.119±0.027
Controls	all	3.05±0.08	67.00±0.86	0.45±0.017	

Significantly more dry matter was accumulated in new leaves at 9,000 lux than at lower light intensities. Tagged leaves did not differ in area from those *in vitro* and did not increase in size significantly with time in soil. However, there was a significant decline in FW/DW and % WC between tagged leaves at the time of transplanting and one month later. This was not affected by the light intensity at which transplants were kept in soil. Plantlets and tagged leaves of transplants at 3,000 lux had significantly lower g DW/dm^2 than at 6,000 and 9,000 lux. While little dry matter accumulated in tagged leaves of transplants at 3,000 lux, there was a significant increase at 6,000 and 9,000 lux.

Discussion

Leaves formed *in vitro* are anatomically (Donnelly and Vidaver, 1984a) and as these results show, physiologically affected by the culture environment compared to control leaves. Light intensity influenced pigment contents and dry weight accumulation of cultured leaves but these factors appeared not to influence a low capacity for autotrophic metabolism. Some anatomical elaborations were noted in the leaf anatomy of plantlets incubated at higher light intensities. These cultures possessed some leaves with a "normal" complement of abaxial filiform trichomes, absent from the leaves of lower light intensity cultures and a degree of compactness of the

parenchymatous leaf tissues marginally greater at higher incubation intensities (Donnelly and Vidaver, 1984a).

Surviving leaves from culture, on transfer to soil, undergo little apparent change. Tagged leaves from 3,000 lux cultures showed either no improvement or a decrease in autotrophic function over a 4 week period. These leaves were net respirers by 1 month after transplant. Tagged leaves did not increase significantly in total area, they did accumulate more dry matter at the higher light intensities.

Features common to cultured plant leaves persist in new leaves appearing after transfer to soil (the transitional leaves). This situation may be analagous to that found by Schmidt and Millington (1968), who reported series of transitional leaf forms produced in Proserpinaca palustris subjected to abrupt environmental change. Thus the transitional leaves may reflect the influence of in vitro conditions on leaf primordia formed in culture as well as the influence of the new ambient environment. Environmentally produced, or epigenetic anatomical features induced by the culture environment, are shown here to have accompanying physiological responses. Leaves with transitional anatomy possessed a transitional CO2 ability. Compared to leaves of transplants at lower light intensities both total leaf area and dry matter content were significantly higher in new leaves formed at 9,000 lux. These leaves had pigment contents equivalent to young control leaves yet they photosynthesized at a lower rate. Carbon dioxide uptake was not

different in transplants grown at 6,000 and 9,000 lux, so was not limited by light. As the time in soil increased subsequently formed leaves tended to become more like controls and higher light intensity given on transplant might accelerate this transition. Complete adjustment to growth in soil appears to be a developmental process requiring the formation of leaf primordia uninfluenced by the culture environment.

Our data clearly indicate that the environmental conditions of culture profoundly influence the anatomical and physiological characteristics of plants propagated by axillary bud proliferation. Development *in vitro* was influenced by exogeneous hormones, the organic carbon source, and the necessarily high humidity, relatively low light intensity and restricted gas exchange. The present study shows that *in vitro* red raspberry has a minimal capacity for autotrophic metabolism. Part of the cause of the low CO_2 assimilation in cultured plantlets is due to the higher photorespiration rates *in vitro*. Control levels of autotrophy are not reached by leaves that develop in the soil environment but that were initiated in culture, and may depend on leaves arising from primordia which originate after transplantation. Thus there appears to be an inherent limitation in the efficiency of the widely employed transplantation protocol. Transplants must undergo a period of acclimatization, more specifically, a period of transitional development in which both anatomical characteristics and physiological performance escape the influence of *in vitro* culture conditions.

IV. Labeled Gas Exchange of Red Raspberry in Culture and transferred to soil

Introduction

Axillary shoot proliferation of *in vitro* plantlets is commonly used to generate identical aseptic specimens for cultivation (Murashige, 1977,1978), yet little is known about how tissue cultured plants are re-established *in vivo* or how transplants acclimatize to soil environments. Evaluation of physiological changes taking place during acclimatization is prerequisite to comprehension of this process and necessary to the development of more efficient transplant protocols. This is important because ..."the ultimate success of plant tissue culture as a commercial means of plant propagation depends on the ability to transfer plants out of culture on a large scale, at low cost, and with a high survival rate"... (Connor and Thomas, 1981). Both leaf anatomy and carbon fixation ability of cultured cauliflower (*Brassica* sp.; Grout and Aston, 1977a,b and 1978) and red raspberry plantlets (*Rubus idaeus* L.; Donnelly and Vidaver, 1984a,b; also see Chapters I-III) are affected by the *in vitro* environment. Cauliflower transplants apparently do not demonstrate net CO_2 uptake until 2 weeks after transfer to soil. New leaves formed the second week after transplant apparently

exhibit control levels of CO_2 uptake (Grout and Aston, 1977b). After transplant, red raspberry is capable of net CO_2 uptake, and developing new leaves are transitional in the sense that both anatomy and photosynthetic CO_2 uptake (of new leaves tested in a group) are intermediate between those of plantlets *in vitro* and greenhouse control plants (Donnelly and Vidaver, 1984a,b; also see Chapters I-III). To further investigate acclimatization in red raspberry, the photosynthetic capabilities of the persistent leaves retained from culture (unlike cauliflower, red raspberry transplants retain these leaves for many weeks) and successively formed new (transitional) leaves were examined; $^{14}CO_2$ was used to quantify uptake 5 weeks after transplant. Coloured markers were used to differentiate between leaves formed in culture and those developing in successive weeks after transplant.

Materials and Methods

Eight plantlets with all leaves tagged were transferred from 2,000 lux culture to each of three light intensities in soil: 3,000, 6,000 and 9,000 lux. At weekly intervals all newly emerged leaves of transplants were tagged with petiole markers of an identifying colour. After 5 weeks in soil transplants from each light treatment were used for labelled CO_2 fixation experiments. These now had markers of six different colours indicating periods of leaf formation.

All raspberry specimens; plantlets, transplants and control plants; were subjected to the same $^{14}CO_2$ fixation assay. The experimental apparatus consisted of a closed gas circuit, with a plant chamber (Voznesenskii, Zalenskii and Austin, 1971). This circuit incorporated a gas generation vessel, gas mixing chamber, Geiger counter and pump. The counter was connected to a chart recorder. Thirty ul of ^{14}carbonate solution of 12 uCi total activity was placed in a gas generation receptacle, and 2.5 ml of 2N H_2SO_4 added to release the $^{14}CO_2$ into a gas circuit excluding the plant chamber. The pump circulated the gases through the mixing chamber, for 5-10 minutes, until an equilibrium maximum, of $^{14}CO_2$ indicated by the Geiger counter trace, was reached. The specimen was inserted into the plant chamber and exposed to cool white fluorescent light of saturating (20,000 lux) intensity. After 1 minute of light exposure the circuit was made to include the plant chamber for a $^{14}CO_2$ exposure period of 5 minutes (±10 seconds). The specimen was then removed from the chamber and its leaves segregated on the basis of the petiole markers. A paper punch was used to take tissue samples of known area (0.385 cm^2) from the many tiny leaves of cultured plantlets and transplants and the large control leaves. When leaves or buds were smaller than the paper punch disc size their outlines were traced onto graph paper for an area estimation. Samples were placed in calibrated test tubes containing approximately 5 ml of hot 80% ethanol. The tubes were capped with aluminum foil and boiled for 2 minutes in a water

bath. The extracts were rapidly cooled by placing the tubes on ice, then made up to 5 ml volume with 80% ethanol. The tubes were agitated and three 100 ul samples withdrawn from each for ^{14}C counting in a Beckmann liquid scintillation counter. Total activity counts were corrected for background activity, then adjusted for volume of the alcohol extract and sample leaf area (Voznesenskii et al., 1971).

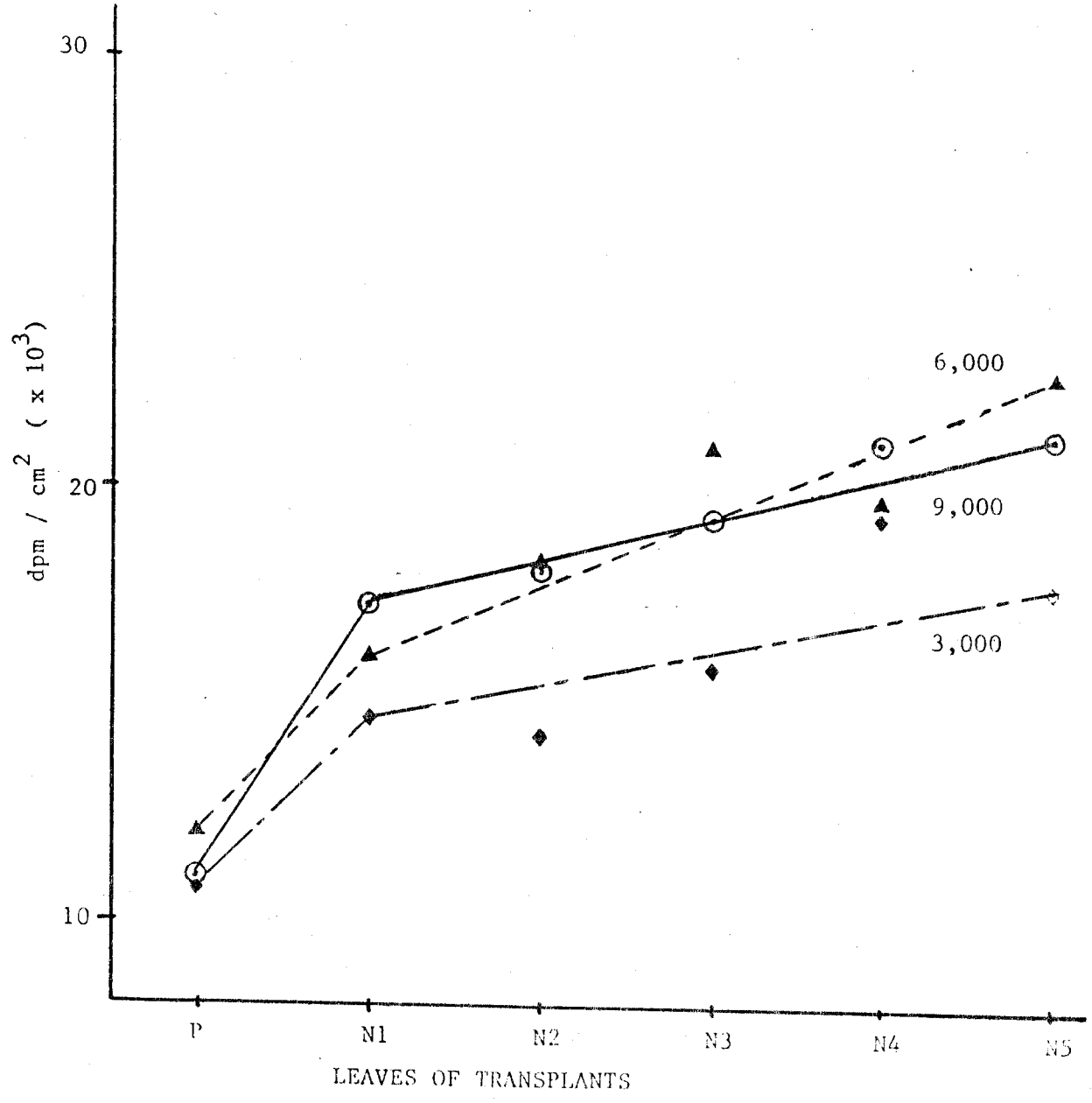
Results and Discussion

Mature control leaves (n=21) tested through the course of the summer gave activity counts that averaged 34,006±1,585 dpm/cm². Young leaves and leaf buds (n=24) gave activity counts averaging 9,415±711 dpm/cm². Leaves of 2,000 lux plantlets, tested at midday directly from their culture containers, had activity levels of 21,149±1,006 dpm/cm². This was higher than expected, suggesting a CO2 uptake rate greater than observed with gas exchange experiments. Carbon dioxide uptake measured over a longer interval in an infra-red gas analyzer showed that cultured plantlets had CO2 uptake rates of only 2-3 mg/dm²/hour compared to control plant rates of 10-15 mg/dm²/hour (Donnelly and Vidaver, 1984b; also see Chapter III). This elevated level of apparent CO2 uptake is most probably brief. Possibly plantlets photorespire non-labeled CO2 of glycolytic, Kreb cycle origin while control plants (and transplants) photorespire labeled CO2.

Measured 5 weeks after transplantation, leaves formed during the first week in soil had activity levels that were much higher than those of persistent leaves (Fig. 6). The most dramatic increase occurred at 9,000 lux, where there was a 160% increase. There was an increase of 135% in 3,000 and 6,000 lux transplants. Carbon dioxide fixation levels at all light intensities were higher in leaves developed during each successive week in the soil environment. The newest growth, developed during the fifth week, achieved activity levels ranging from 165% of culture-formed leaves in the 3,000 lux transplants to almost 200% in those at 9,000 lux. Maximum activity levels of new leaves were 53-66% of that of mature greenhouse control leaves, presumably much closer to plants grown at comparable light intensities. There appears to be an advantage to transplanting to the highest light intensity (9,000 lux): these transplants had the greatest activity levels and leaves formed during the first week in soil at 9,000 lux had the greatest increase in CO2 uptake ability over the persistent leaves. Furthermore, it has been demonstrated that leaf area and dry matter content are significantly higher in new leaves formed at 9,000 lux (Donnelly and Vidaver, 1984b; also see Chapter III). The graded increase in CO2 uptake seen in successive week's growth at all light intensities is apparent despite the fact that each successive week's growth is of a younger age class. It is clear that during acclimatization photosynthetic carbon fixation capacity increases as leaf anatomy undergoes

transition away from the effects of culture and this is accelerated by higher transplant light intensities.

Figure 6. $^{14}CO_2$ uptake in leaves of transplants (persistent (p) and successive weekly age classes of new (n) leaves) grown at 3 light intensities (3,000, 6,000 and 9,000 lux) and tested at 20,000 lux.



V. Starch Content, Chlorophyll a Fluorescence Induction and Oxygen Uptake of Red Raspberry Plantlets and Control Plants

Introduction

The capacity of plants to store starch in their leaves, their chlorophyll a fluorescence induction kinetics and the photochemical activities of extracted chloroplasts can all be measures of photosynthetic capability. Virtually nothing is known of the starch storing capacity of cultured plantlets or the fluorescence induction kinetics of their leaves. There have been no reports of chloroplast extractions from plantlets propagated by axillary bud proliferation, or measurement of photosystem I and II activities. Tests were done on cultured red raspberry plantlets to acquire a better understanding of the effects of culture on their photosynthetic activity. There is an historical aspect to the starch evaluations tests. Wardle, Quinlan and Simpkins (1979) suggested that leaves from culture had a food storage function after transplant in regenerated plantlets of Brassica oleracea var botrytis cv. Currawong. This "food storage hypothesis" was based on the presence in these leaves of "large quantities of reserve starch" (A. Quinlan, unpublished; Wardle et al., 1979). To test their hypothesis Wardle, Dalsou, Simpkins and Short (1983) attempted to trace the

movement of rubidium, applied in the culture medium as RuCl, in the leaves of transplants. They were able to demonstrate some movement of label from the leaves persisting from culture to the new leaves formed in soil. Evidence that Ru^{+} movement from cultured leaves to the new leaves of transplants supports their food storage hypothesis is not convincing because the mere withdrawal of ions from these leaves as they senesce does not lend these tissues (the leaves from culture) a food storage function. If leaves from culture were shown to contain starch in large quantities that was demonstrably depleted by new growth then they could justifiably be termed food storage organs. Furthermore, conditions of plantlets prior to transplant that tended to increase starch storage should improve transplant performance if this hypothesis is correct.

Materials and Methods

In order to determine the applicability of the food storage hypothesis to red raspberry plantlets the amount of leaf starch was measured and related to the results of transplanting 36 plantlets from each of 2 light intensities in culture (2,000 and 6,000 lux). All starch tests on control plants and cultured plantlets incubated at 2,000-6,000 lux were done on detached leaves or leaf segments harvested at midday. Samples were decolourized in warm 95% ethanol and submerged in a solution of IKI for 10 minutes or more.

Chlorophyll a fluorescence induction determinations were made at room temperature using leaf sections of dark adapted (minimum of 1 hour) plantlets and plants by a procedure developed by Schreiber, Fink and Vidaver (1977). Leaf samples were obtained from mature leaves of red raspberry plants grown in the greenhouse, and from tissue cultured plantlets incubated at 2,000, 4,000 and 6,000 lux. These leaf sections were centered over the plexiglass sample window, abaxial side down, and enclosed in the sample compartment. All determinations were performed using the same procedure: a brief flash of about 80 ms for an O-level determination; then after an interval of 1 minute darkness, a 15 seconds illumination period to determine the induction time course. This was followed by removal of the sample to a solution of 10^{-5} M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) for 1 hour in the dark, then by resampling of the inhibitor-treated disc. The fluorescence apparatus consisted of a 650 watt tungsten iodine lamp shielded by a Corning 4-96 (blue) filter, and adjusted to 1 mwatt/cm^2 at the sample. A bifurcated fibre optics system led excitation light through one arm to the sample and fluorescence was detected by an EM1 9558QB photomultiplier through a Corning 2-64 (red) filter in the other fiber optics arm. The photomultiplier was operated at 800 volts and its output stored in the memory of a Tracor Northern TN1710-30 signal averager, printed out with a Hewlitt-Packard X-Y chart recorder. Light flashes were controlled by means of a Compur Electronic shutter

that had a 2 ms opening time. This permitted reasonably precise O-level measurements.

Chloroplasts from tissue cultured red raspberry plantlets incubated at 4,000 lux and greenhouse control plants were prepared from 25 g of leaf tissue by a procedure modified from Cohen, Popovic and Zalic (1979). For each isolation tissue was ground in a blender in medium consisting of 0.33 M sorbitol, 0.5 mM MgCl2, 0.4% (w/v) PVP-40 (polyvinylpyrrolidone) to inhibit phenyloxidase reactions and 10 mM phosphate buffer ($Na_2P_4O_7.H_2O$) adjusted to pH 6.5. The homogenate was filtered through many layers of cheese cloth, resulting in ca. 25 um pores, and centrifuged at 3,500 g for 5 minutes. The chloroplast pellet was resuspended in buffered medium containing 0.33 M sorbitol, 2 mM EDTA (ethylenediaminetetraacetic acid), 1 mM MgCl2, 1 mM MnCl2, 50 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesolfonic acid) at pH 7.6, then recentrifuged and again resuspended. Photosystem II (PSII) and I (PSI) activity were measured using a Clark-type oxygen electrode, by the methods of Cohen et al. (1979) and Delieu and Walker (1972). To measure PSI activity, O2 consumption was monitored in a 1 ml chamber holding 3 mM Na isoascorbate, 0.2 mM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine), 0.1 mM methyl viologen, 20 ul chlorophyll, 15 uM DCMU and 910 ul buffer. Photosystem II activity was measured by monitoring O2 evolution in a 1 ml chamber holding 100 ul chlorophyll, 830 ul buffer, 1.5 mM FeCN and 2.5 mM NH_4Cl.

Results and Discussion

Control leaves exposed to iodine solutions turned blue-black very rapidly, demonstrating large amounts of stored starch. Red raspberry plantlets showed total or almost complete absence of starch in some leaves, in cultures from all light intensities, and various amounts, often distributed in a non-uniform way in others. Higher light intensity cultures generally had more starch. Transplant success from high light intensity cultures has been demonstrably poorer than from low light intensity cultures. For example, while 33/36 transplants from 2,000 lux culture survived, only 5/36 transplants from 6,000 lux did so (see Chapter II). Photoinhibitory phenomena (discussed by Bjorkman, 1981) cause failure to produce starch as well as disappearance of starch from leaves. There may not necessarily be any associated change in leaf chlorophyll content. Inhibition of photosynthesis preceeds changes in chlorophyll and starch contents and may be caused by excess light or restricted CO2 or O2. Severe restriction of CO2 supply to an illuminated leaf such as would occur under conditions of stomatal closure, or *in vitro* despite the open stomata, inevitably results in reduction of CO2 partial pressure inside intercellular spaces of the leaf and in a drastic reduction in photosynthetic rate. This increases the susceptibility to photoinhibition by several fold (Bjorkman, 1981). Despite the

presence of glucose in the medium, it is apparent that starch synthesis is governed by photosynthetic sugar production, and is not merely the consequence of sugar availability. Photoinhibition is a likely consequence of presumed CO2 depletion in the culture containers and results in the inhibition of starch synthesis.

Chlorophyll a fluorescence induction analysis has been used diagnostically and may have further uses in the evaluation of tissue cultured material (Schreiber, Fink and Vidaver, 1977). Levels of P-O/O (O-levels were normalized to 1) for plantlets are two thirds of control levels and are affected little by culture light intensity (Table 6). The steepest DP rise slopes occurred in plantlets incubated at 4,000 lux (Table 6). Incomplete penetration of DCMU was suspected in some samples from the shape of the DCMU curves so data were recalculated using the subsample where DCMU curves indicated complete sample penetration (Table 6). Of interest was increasing DCMU penetrability in the order (presumably related to leaf anatomy) of controls, 6,000 lux, 4,000 lux and 2,000 lux plantlets.

Rates of oxygen evolution (PSII) and consumption (PSI) have been used diagnostically and may be useful in the assessment of photosynthetic capability of cultured plantlets. When extracted chloroplasts were evaluated it was possible to maintain activity for close to 1 minute before inhibition. During this period control levels of PSII oxygen evolution were 60 uM O2/mg chlorophyll/hour. Cultured tissue PSII had oxygen evolution

rates of 20 uM/mg chlorophyll/hour, or one third of control levels. This approximated CO2 uptake ability seen in intact plantlets, 2-4 mg CO2/dm²/hour, compared to control rates of 12-15 mg CO2/dm²/hour, (Donnelly and Vidaver, 1984b; also see Chapter III). Photosystem I oxygen consumption rates were 1,420 uM O2/mg chlorophyll/hour for the cultured tissue, and far lower than expected, ca. 100 uM O2/mg chlorophyll/hour for the controls. These extractions and measurements were quite difficult and time consuming for routine use but they yield alot of information, can be done in conjunction with pigment quantifications and can probably be scaled down to minimize the amount of cultured material utilized.

Table 6. Chlorophyll a fluorescence induction analysis of red raspberry plantlets at three light intensities in culture and greenhouse control plants.

Light intensity (lux)	n	DP slope	P-O/O		
			Control	n	DCMU
Culture Soil					
Plantlets 2,000	10	0.62±0.13	1.92±0.23	7	2.36±0.15
4,000	11	1.75±0.12	2.15±0.13	3	2.28
6,000	10	1.15±0.11	1.98±0.20	5	2.06±0.26
Controls	10	0.67±0.08	2.84±0.11	1	3.07

VI. Spent Medium Assessment; a Potential Analytical Tool in Culture Improvement

Introduction

Historically, decisions to subculture tissue cultured plantlets to new medium or transplant them to soil have been based on a limited number of criteria including number of survivors (stage I, stage IV), number of axillary shoots (stage III) and general culture appearance. These decisions have never been based on knowledge of medium depletion. Furthermore, while decisions involving environmental or medium parameter adequacy or optima have sometimes been based on fresh or dry weight accumulation in cultured tissues, they have not considered efficency of utilization of medium components. Yet medium examination should hold clues to plantlet performance *in vitro* and is a likely source of comparative data for accurate growth evaluation. In this study the effects of varying one important environmental parameter (light intensity) and the simplest medium parameters (medium volume and dilution) were examined. Media were investigated, following five 4 week culture cycles of red raspberry, for changes in total volume, pH, osmolarity and in some cases, glucose concentration.

Materials and Methods

Cultures were incubated at 5 light intensities, from 2,000 to 6,000 lux. At 2,000 and 3,000 lux 2 medium volumes were employed, 30 and 40 ml per culture container. At 4,000-6,000 lux 40 ml of medium was used in each culture container. All cultures were treated identically; incubation temperature was 27 ±2 °C, photoperiod was 16 hr light:8 hr dark. Cool and warm white fluorescent tubes were used as the light source at a ratio of 3 cool:1 warm. Light intensity was achieved by adjusting the culture distances from the light source. Plantlets were subcultured at 4 week intervals. Subdivision was done as equally as visually possible; five 2-5 leaf cuttings were placed in each culture jar. On the final culture cycle the effect of starting medium dilution was evaluated at 3,000 lux light intensity on both medium and plantlets. Starting liquid medium volume was 40 ml; medium was full strength or diluted by one quarter, one half or three quarters. Plantlets were examined for leaf fresh and dry weights (minimum 48 hours in a 50 °C oven) and areas. The fresh/dry weight ratios (FW/DW), percentage water content (% WC) and area per gram dry weight (dm^2/g DW) were calculated. Only trends are reported for the medium dilution trial as five or less culture vessels were examined for each medium dilution. The starting pH of the medium was adjusted to 5.7 and fell to 5.5 after autoclaving. The osmolarity of new medium was 218-219 mOs/L; the bulk of this was glucose at 25g/L. Medium (undiluted)

volumes of 30 and 40 ml per jar initially had 6.56 and 8.74 mM of sclute per jar (4.20 and 5.60 mM of glucose) respectively.

Medium was examined over a 5 month period following routine monthly subculture of plantlets. Medium volume was measured in a graduated cylinder. Medium volume utilized was calculated from dozens of harvested cultures, incubated at five light intensities, used for gas exchange measurements (see Chapter III), multiplied by the percentage water content. The balance of the medium volume change at the end of each month culture is due to evaporation from the containers. The pH was tested with a Fisher accumet model 425 digital pH/ion meter. Osmolarity was measured with a Wescor model 5100A digital vapor pressure osmometer. Glucose measurements were made by a colourometric enzymatic determination based on the following coupled enzymatic reactions:

1. Glucose + $2H_2O$ + O_2 $\xrightarrow{\text{glucose oxidase}}$ Gluconic acid + $2H_2O$
2. H_2O_2 + o-Dianisidine (clear) $\xrightarrow{\text{peroxidase}}$ o-Dianisidine (brown)

Absorption was measured spectrophotometrically at 425-475 nm and was proportional to the glucose concentration. This test consistently underestimated the amount of glucose present, possibly due to interfering substances in the medium. By shortening the suggested incubation interval of 45 minutes, the effect of interfering substances was minimized.

Univariate one-way analysis of variance and Scheffe Allowances (among the five light intensity treatments), and t-tests or Mann-Whitney U tests (for the two medium starting

volumes) were used to determine if the means were significant at the $p \leq 0.10$ level for the multiple comparisons, otherwise at the $p \leq 0.05$ levels (Kleinbaum and Kupper, 1978).

Results and Discussion

The decrease in medium volume per culture container did not vary with the light intensity of incubation but did vary with the starting volume of the medium. There was a significantly greater decrease in medium volume when the starting volume was 40 ml than when it was 30 ml. A large portion of this is due to evaporation (Table 7). The medium pH decreased ca. 1 pH unit during 1 month culture but did not vary with the light intensity of incubation or the starting medium volume (Table 7). Although mean water potential showed a trend towards the more negative, from 6,000 lux to 2,000 lux culture, among the 40 ml starting volume cultures, this difference was not significant. Starting medium volume did have a significant effect on the mean water potential; more solute was utilized per culture jar when the starting medium volume was 40 ml than when it was 30 ml (Table 7). These results were reflected in the most abundant solute, glucose, utilized. After 1 month culture there was no difference in glucose used among the 4 light intensities tested (6,000 lux culture medium was not adequately tested). There was a significant increase in absolute glucose utilization in cultures with the greater starting volumes; 142 mg more utilized at 2,000

lux, 207 mg more utilized at 3,000 lux. However, the amount utilized at 2,000 lux (65-68%) and 3,000 lux (81%) as a percentage of total available glucose did not change with starting medium volume (Table 7).

In the medium dilution trials where the starting medium volume was kept constant (40 ml), there was no difference in the mean volume of medium utilized or the final pH of the medium (Table 8). The amount of solute used was greatest in the jars containing full and three quarter strength medium (Table 8). These jars also possessed leaf tissue with the greatest amount of dry matter accumulation per unit area (Table 8).

Some decisions can readily be made on the basis of these tests. It is clear that 40 ml was a superior starting medium volume than 30 ml. Perhaps, in the case of starting medium volume more is better; this should be further tested. In 40 ml cultures more medium and more solutes, especially glucose were removed during culture than in 30 ml cultures. With a larger starting volume, the same amount of solute uptake as from a smaller volume results in a less positive water potential and molar concentrations of solutes would decrease less rapidly. Full or three quarter strength medium was preferable to medium that had been further diluted. The best light intensity of incubation was not determined conclusively since it was not possible to distinguish between them on the basis of amount of medium utilized nor solute utilized. However, among the 4 light intensity cultures tested, the most glucose was utilized at

3,000 lux and this intensity would be a likely candidate if light intensity was to be standardized for further tests. In conclusion, "spent" medium evaluation would appear to be a hitherto overlooked but valuable tool in tissue culture propagation. Its use could contribute to and objectify decisions involved in medium optimization and environmental parameter adjustment.

Table 7. Red raspberry culture medium (means and standard errors tested after 1 month at 5 light intensities (2,000-6,000 lux) and 2 starting medium volumes (30 or 40 ml).

Light intensity (lux)	Volume (ml)			pH decrease	Solute utilized (mM)		
	Initial	Final (decrease)	Used		Total	Glucose	Other
	40 30						
6,000		12.68±0.69	1.62	0.99±0.08	5.11±0.42		
5,000		10.83±0.76	1.50	0.97±0.07	4.11±0.40	3.57±0.28	0.54
4,000		11.62±0.44	2.51	0.94±0.04	4.25±0.27	3.60±0.51	0.65
3,000		11.36±0.35	2.12	0.89±0.09	4.11±0.23	3.90±0.31	0.22
	3,000	9.70±0.54	1.92	0.89±0.04	2.86±0.18	2.82±0.11	0.04
2,000		11.33±0.92	1.18	0.71±0.06	4.11±0.26	3.77±0.18	0.35
	2,000	8.03±0.53	1.17	0.79±0.05	2.88±0.18	2.45±0.34	0.43

Table 8. Red raspberry culture medium and plantlets (means and standard errors) tested after 1 month at 3,000 lux and 40 ml starting volume with 4 starting medium dilutions (4/4, 3/4, 2/4 and 1/4 strength).

Medium strength	Volume used (ml)	pH decrease	Solute used (mM)	Plantlet g DW/dm²
4/4	1.35±0.05	0.89	2.49±0.27	0.22±0.06
3/4	2.19±0.41	0.64±0.03	2.41±0.42	0.18±0.01
2/4	1.83±0.15	1.18±0.06	1.35±0.24	0.14±0.01
1/4	1.81±0.27	0.91±0.02	0.51±0.14	0.14±0.02

D. Summary and Conclusions

Leaves of red raspberry plantlets formed in culture were almost always unifoliate, smaller, thinner, had a less compact arrangement of palisade and mesophyll cells, and an altered epidermal and palisade cell shape, compared to leaves formed on plants in soil. The epidermal cells were collapsed to some extent, the palisade cells obconical; unlike the rectangular control leaf palisade cells. The ratio of palisade:epidermal cell numbers was usually 1:1 in cultured leaves, many:1 in controls. Despite the changes in leaf and cell sizes and shapes, the ratio of cell and tissue widths to leaf widths (defined in the adaxial-abaxial plane) was similar in leaves of plantlets, transplants and controls. The number of epidermal hairs, especially the filiform type, was lower *in vitro* and the distribution of colleters was affected. In cultured plantlet leaves colleters alternated with straight or falcate unicellular hairs on the leaf edges. In controls the leaf edges were densely covered with straight or falcate hairs; these entwined with abaxial filiform hairs. Trichome number was greater in new leaves formed after transplantation, and greatest in greenhouse and field grown control plant leaves. Stomata were fixed open (and apparently incapable of closing), guard cells were slightly raised and occurred both on the lower and the upper leaf surface of *in vitro* plantlets with many on the periphery of the leaf. Calcium oxalate crystals were present in the leaves of *in vitro* plantlets and more numerous in the leaves grown on plants in soil. The petiole, leaf, stem and root anatomy of *in vitro*

plantlets were similar in low (2,000 lux) and high (6,000 lux) light intensity cultures. These had no sclerenchyma, little or no collenchyma support tissues, little secondary wall deposition and were much smaller. Cultured plantlets demonstrated relatively low levels of CO2 uptake, averaging 2.5 mg $CO2/dm^2$/hour and rarely exceeding 4 mg $CO2/dm^2$/hour at saturating light intensities, compared to control plant rates of 12-15 mg $CO2/dm^2$/hour. Pigment content was higher in plantlets incubated at lower light intensities (2,000-4,000 lux) than higher ones (5,000-6,000 lux). Starch deposition was inhibited in leaves of plantlets in culture, and photosystem II activity of extracted chloroplasts was only one third of normal.

New leaves formed during the first month in the soil environment were usually unifoliate, intermediate in size and thickness and had the loosely arranged cells of the in vitro plantlets, and at the lower light intensities, some of the external leaf characteristics. When cultured plantlets were transferred to soil at 3,000 or 6,000 lux, the amphistomatous and peripheral stomata persisted as did the elevated stomatal condition in new leaves during the first month. However, at 9,000 lux new leaves, like all greenhouse and field control leaves, had few adaxial stomata, peripheral stomata were rare and guard cells were usually flush with the epidermal cells. Anatomy of new leaves formed during the first month after transplanting in soil at 3,000, 6,000 or 9,000 lux was similar to those in culture. Parenchyma cells were less compact than in

control plant leaves and palisade cell shape remained abnormal. More than half the leaves from culture died within the first month of transferring plantlets to soil. Some survived for almost 3 months. Reduced trichome numbers, almost complete lack of filiform trichomes and presence of peripheral and adaxial as well as unprotected, open, abaxial stomata would all contribute to transplant shock and water loss in cultured plantlets transferred to soil. Transplants had intermediate amounts of secondary wall thickenings and sclerenchyma and collenchyma support tissues in all organs, and some periderm development in stems and roots. Persistent leaves after 1 month in soil accounted for 30% of the total leaf area of transplants but contributed less than 10% of the CO2 uptake at 3,000 lux. At higher light intensities these leaves accounted for 10 to 30% of the total leaf area but were net respirers. There was an increase in dry matter accumulation at 6,000 and 9,000 lux in these persistent leaves, but not at 3,000 lux. Continued accumulation of dry matter by the tagged leaves was probably at the expense of photosynthetic activity of the newly formed leaves. New leaves of transplants had a greater dry matter accumulation at 9,000 lux, and a pigment content greater than the persistent leaves. Their pigment content was similar to that of young control leaves. Transplants were capable of uptake rates of 5-7 mg $CO_2/dm^2/hour$ or 50% of field control rates. The photosynthetic contribution of the leaves from culture was small or negative. The first new leaves formed in soil were

transitional with intermediate capability. New leaves of transplants, tested after a 5 week period in soil, showed progressive ability to take up labeled CO2 in each successive week's growth. This is illustrative of acclimatization of transplants to the soil environment. It demonstrates that as new leaves are produced by transplants they display a spectrum (progression) of physiological activity, gradually increasing from levels close to that of leaves persisting from culture, to that of control plants.

Relatively simple tests performed on spent medium, such as measurement of medium volume remaining and pH, osmolarity and glucose concentration, enabled straightforward selection among two starting medium volumes, four medium dilutions and five light intensities employed in the culture of red raspberry plants. These results confirmed those of dry weight accumulation in the leaves of cultured plantlets but were non distructive of cultured material and much easier to perform.

The term epigenetic describes changes in the phenotype and involves the expression of various genes, not changes in their composition. The normal growth and ontogenetic patterns of plants involve progressive epigenetic changes, the rate of which are related to the number of cell divisions (Hartmann and Kester, 1983). Unlike purely physiological changes, epigenetic changes persist when a particular stimulus ends. Mechanisms of epigenetic control are thought to involve the t-RNA system of relaying genetic information to appropriate enzyme systems

(Hartmann and Kester, 1983).

Normally structural change is adaptive to the ambient environmental pressures. That this is true of tissue cultured red raspberry plantlets is known from their phenomenal propagation potential in vitro (Donnelly, 1980; Donnelly, Stace-Smith and Mellor, 1980). In the case of propagules coming through the micropropagation protocol transplantation to soil involves an abrupt environmental change and leads to transplant shock. The phenomenon of transplant shock is a direct resultant of maladaptive structural and physiological changes induced by the culture invironment (epigenetic variation), suitable therein, but inappropriate to life in soil.

During the acclimatization phase (stage IV) new transplants look quite different from control plants. They display a variety of anatomical modifications, (see Table 1) some more obvious and potentially injurious than others. In addition they are the subject of physiolcgical changes induced by the in vitro environment. The physiological changes are reversible in whole or in part insofar as the modified anatomy can support these reversals. During the acclimatization process these atypical anatomical and physiological modifications are lessened in the new leaf growth, and with time and complete acclimatization they are no longer apparent in subsequent growth. New growth in soil environments with relatively low light intensity and elevated relative humidity (transplant conditions) is a) likely to exhibit the features of cultured plants induced by the low light

intensity and saturated atmosphere *in vitro* b) if leaves were initiated in culture, and are expanding in soil, they may look more like leaves *in vitro* than do new leaves initiated in the soil environment c) initiation in soil will give phenotypes that reflect the ambient environmental conditions present during organogenesis and the physiological status of the rest of the plant; this implies that return to control-type anatomy most likely progresses in step-wise fashion. This may explain the intermediate-type anatomy observed in well established red raspberry transplants. The physiology will vary in these plants a) it may be to some extent limited by continued low lighting and high relative humidity, if this is a stimulus for altered physiology *in vitro* b) the altered phenotype may exacerbate physiological problems and cause them to be corrected more slowly than otherwise predicted, again probably in step-wise fashion. This may explain the low CO2 fixation rates observed in transplants many weeks after transplantation, much longer than apparent leaf anatomy would indicate.

There is little doubt that epigenetic modifications are induced by the *in vitro* growing conditions. As documentation increases of anatomical and physiological changes in stage IV plantlets (see Table 1), it beccmes more apparent that subjective evaluation of plant morphological development (Street and Shillito, 1977) should be replaced by direct diagnosis of anatomical and physiological competence in cultured plants both *in vitro* and after transplantation. Functional criteria should

be applied to stage III plantlets (eventually even at stage I and II) as diagnostic tools in media and environmental parameter improvement, to increase both stage IV survival and transplant performance. By compensating for the effects of specific environmental factors on plantlet development, plantlets in culture could be produced that are more likely to survive and more productive on transplantation. In addition, transplants would be treated in such a way as to minimize the residual effects of culture, and maximize their performance in soil.

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