ACCLIMATIZATION OF MICROPROPAGATED 'SILVAN' BLACKBERRY

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ACCLIMATIZATION OF MICROPROPAGATED 'SILVAN' BLACKBERRY

ABSTRACT

M.Sc. Laurence Tisdall Plant Science

Acclimatization of micropropagated
'Silvan' blackberry.

Tissue-cultured shoots and plantlets usually have leaves with non-functional, open stomata and little epicuticular and cuticular wax, resulting in excess evapotranspiration after transplantation. Various strategies were evaluated to decrease ex vitro acclimatization difficulties for 'Silvan' blackberry, including transplanting unrooted shoots, increasing the medium agar concentration from 6 to 9 or 12 g/l and diluting the basal medium. Increased medium agar concentrations and medium dilution did not improve survival or growth. Stomatal function resumed sooner in new leaves of plantlets than shoots. High relative humidity (> 95 %) and low light intensity (90 μmol s^{-1} m^{-2}) negatively affected stomatal closure both on acclimatizing transplants and greenhouse-grown plants. Guard cells developed on leaves in vitro were physiologically active but had apparent anatomical abnormalities that inhibited closure. A rapid clearing and staining method was developed for examination of foliar morphology using intact in vitro blackberry (Rubus sp. 'Silvan') and strawberry (Fragaria x ananassa Duch. 'Totem') plantlets and sections of greenhouse-grown 'Silvan' and 'Totem' leaves. This method involved three steps: 1) removing the chlorophyll by autoclaving in 80 % ethanol; 2) dissolution of the protoplasm using 5 % NaOH at 80 °C; 3) post-alkali treatment with 75 % bleach (4.5 % NaClO) at room temperature for tissue-cultured plantlets and at 55 °C for greenhouse-grown leaves. Aqueous safranin (10 mg/l) was used for staining.

RESUME

M.Sc. Laurence Tisdall Phytotechnie

Acclimatation de la mûre 'Silvan' sp. micropropagée

Les pousses et les plantules provenant de la culture de tissu ont généralement des feuilles sur lesquelles les stomates ne fonctionnent pas et sont ouvertes ainsi que une formation incomplète ou anormale des cires épidermiques. Ceci s'aboutit à un manque de contrôle sur l'évapotranspiration après leur transplantation. Des stratégies variées ont été évaluées afin de réduire les difficultés d'acclimatation de la mûre 'Silvan' ex vitro, incluant la transplantation de pousses non-enracinées, en augmentant la concentration d'agar dans le bouillon de culture de 6 à 9 ou 12 g/l et en diluant le bouillon de culture basale. Les concentrations d'agar augmententées et la dilution du bouillon n'ont amélioré ni la survie ni la croissance. Le fonctionnement du stomate a repris plus tôt pour les nouvelles feuilles des plantules que celles des pousses. Une atmosphère presque saturée (> 95 %) et une intensité de lumière basse (90 µmol s^{-1} m^{-2}) ont affecté négativement la fermeture des stomates sur le plantes provenant de la culture de tissu et les plantes venant de la serre. Les cellules de garde développées in vitro ont fonctionné physiologiquement mais avec des anomalies anatomiques évidentes qui ont rendu impossible la fermeture complète des stomates. Afin d'examiner la morphologie des feuilles, une méthode rapide d'éclaircisment et de coloration a été développée en utilisant des plantules de mûre (Rubus sp. 'Silvan') micropropagées intactes et de fraise (Fragaria x ananassa Duch. 'Totem') en plus des sections de feuilles des plantes 'Silvan' et 'Totem' poussées dans une serre. Cette méthode comprend trois étapes: 1) enlever la chlorophylle en se servant de l'autoclave dans une solution de 80 % d'ethanol; 2) la dissolution du protoplasme en utilisant 5 % de NaOH à 80 °C; 3) un traitement post-alcalin avec 75 % de chlorure décolorant (4.5 % NaClO) à la température de la pièce pour des plantules in vitro et à 55 °C pour les feuilles provenant de la serre. On se servit de la safranine (10 mg/l) pour la coloration.

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ABBREVIATIONS USED

Abbreviation - Common Name	Chemical name
Growth regulators	
ABA - Abscisic acid	[s-(z,E)]-[5-(1-hydroxy 2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pent-adienoic acid
BAP - Benzylaminopurine	N-(phenylmethyl)-1H-purin-6-amine
IBA - Indole-3-butanoic acid	1H-indole-3-butanoic acid
Fungicides	
Benlate^{R} - Benomyl	1-methyl[1 [(butylamine)carboxyl-1H benzimidazol-2yl] carbamate
Truban^{R} - Ethazole	E-5-ethoxy-3-(trichloro ethyl)-1,2,4-thiadiazole
Fertilizers	
14:14:14	14 N:14 P_2O_5:14 K_2O
20:20:20	20 N:20 P_2O_5:20 K_2O
10:52:10	10 N:52 P_2O_5:10 K_2O
Potting mixture	
Promix^{R}	(1 peat:1 perlite:1 vermiculite)

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perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review.

Concerning the manuscript titled "Acclimatization strategies for micropropagated plants" for the volume "Micropropagation of Woody Plants", the author's contribution consisted of gathering and summarizing all pertinent information as well as submitting a first draft of the review. Subsequent rewriting and editing was done by both authors.

Concerning the publication titled "A rapid clearing and staining method for tissue-cultured plantlets and greenhouse-grown leaves", the author devised and completed the experiment and wrote the manuscript. Dr. Donnelly provided laboratory space and equipment and edited the manuscript.

1. INTRODUCTION

Red raspberry (Rubus idaeus L.) and blackberry (Rubus (Tourn.) L. subgenus Eubatus) are found in temperate zones around the world (McPheeters et al., 1988). Rubus species belong to the Rosaceae family. Red raspberry is the best known and most cultivated of this genus (Snir, 1988). Total world production of red raspberry totals 277 131 mt of which Canada produces 5.4 %. The production of raspberries has more than doubled over the last 20 years, from an average yield in 1961-1965 of 107 006 mt (FAO, 1976; cited in Snir, 1988) to the present 277 131 mt (FAO, 1984). The increase in production is due mainly to enlarged plantation areas and improved yields. Yield increases have resulted from using new cultivars, specific virus tested stocks and new growing and harvesting methods (Snir, 1988).

Blackberry species are native in many parts of the world but little domestication or commercial production have occurred with these except in North America. This is most likely due to the readily available fruit in the wild and the thorny, unmanageable character of wild blackberry plants (Moore, 1984). Until the 1940's, most blackberry acreage resulted from chance seedings from the wild. Most of the virus and virus-like diseases which occur in cultivated and wild blackberry are latent and moderately depress plant vigor and yield. Some diseases can be more serious, causing recognizable symptoms and severely weakening the plants (Converse, 1984).

Although tissue-culturing blackberry is more expensive than conventional vegetative propagation methods, problems with disease may be considered more important than cost considerations (Caldwell, 1984). "Tissue culture may be the only practical method of redistributing virus-free material quickly if strict certification programs for Rubus are put into effect" (Converse, 1981). The tissue culture of blackberry also allows for rapid dissemination of new cultivar releases and year-round production of plantlets (Converse, 1984). Blackberry multiplies very rapidly in culture providing ample clonal

plants (3-6 x every 3-4 weeks) (Kyte, 1987) for production and research purposes.

'Silvan' (Rubus sp.) is an outstanding blackberry cultivar from Australia, with high yield, good fruit quality and increased tolerance to disease compared with other cultivars (McGregor and Kroon, 1984). It is also tolerant to environmental stress such as wind and drought.

Tissue cultured plants have phenotypic characteristics which reflect acclimatization to the unique environmental conditions found in vitro. These phenotypic characteristics include a reduced foliar epicuticular and cuticular wax layer when compared with control, greenhouse-grown plant leaves (Conner and Conner, 1984, Fabbri et al., 1986, Sutter and Langhans, 1982, Sutter 1984, 1985), non-functional, open stomata (Brainerd and Fuchigami, 1981, Donnelly et al., 1986, Short et al., 1987, Wardle and Short, 1983), and a mixotrophic mode of nutrition that relies principally on sucrose as a carbon source (Conner and Thomas, 1982). Together, these characteristics result in limited control over evapotranspiration rates (Brainerd and Fuchigami, 1981, Marin et al. 1988, Sutter, 1988) and initially low rates of photosynthesis in transplants from culture (Grout, 1988). High mortality and severe dehydration may occur after transplantation ex vitro if proper steps are not taken to slowly acclimatize the transplants to the greenhouse or field environment. This generally consists of placing the transplants under conditions of high relative humidity (95-100 %) and relatively low light intensity (approximately 150 μmol m^{-1} s^{-2}), not exceeding three times culture levels (Donnelly and Vidaver, 1984b). Subsequently, the relative humidity is lowered and the light intensity increased over a period of approximately one month. The acclimatization period ex vitro may be reduced when certain in vitro acclimatization strategies are used. These include increased agar concentrations in the culture medium (Ziv et al., 1983) and rooting in vitro (Murashige, 1974).

The objectives of this research were to:

1. Develop a rapid foliar clearing and staining method to enable examination of the external morphology of tissue cultured plantlets and greenhouse-grown plant leaves.

2. Determine the effects of both increased agar concentrations (6, 9 and 12 g/l) in the culture medium and in vitro rooting on ex vitro survival, growth and stomatal characteristics of micropropagated 'Silvan' blackberry shoots.

3. Determine the effect of high relative humidity and low light intensity on stomatal function of greenhouse-grown `Silvan' plants and ex vitro plantlets from full and 1/4 strength modified MS (1962) basal medium.

It is hoped that these experiments will lead to better methods of ex vitro acclimatization as well as an improved understanding of stomatal function in vitro and during the important initial weeks following ex vitro transplantation.

ACCLIMATIZATION STRATEGIES FOR MICROPROPAGATED PLANTS

Danielle J. Donnelly and Laurence Tisdall

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2.1. Introduction

The successful ex vitro acclimatization of micropropagated plants determines the quality of the end product and, in commercial production, the economic viability of the enterprise [6]. When shoots or plantlets are transplanted from culture to greenhouse conditions they may desiccate or wilt rapidly and can die as a result of the change in environment, unless substantial precautions are taken to accomodate them. In commercial micropropagation this step is often the limiting factor [53] and at best, is challenging, labour intensive and costly [6, 7, 10]. Methods which work for ex vitro establishment of one species are not necessarily satisfactory to ensure the survival of another [47].

The following discussion was not intended to be an exhaustive survey of the now extensive literature pertaining to acclimatization of micropropagated plants. The reader is directed to several excellent reviews [6, 7, 10, 39, 75]. We feel that overcoming ex vitro acclimatization problems is contingent on an improved general understanding of how the environment affects the anatomy and physiology of all plants subjected to environmental change. It is necessary to begin with a better understanding of the unique effects of the in vitro and the ex vitro environments on plant phenotype. It is information on this subject that is summarized herein. In this way we hope to provide some new insights into modern acclimatization strategies, applied both in vitro and ex vitro.

2.2. The Culture-Induced Phenotype

Tissue cultured shoots and plantlets share certain characteristic features that are inconsistent with development under greenhouse or field conditions. The culture-induced phenotype (CIP) [16] reflects epigenetic variation [18]; acclimatization to environmental conditions which exist within the closed culture containers. In vitro environments are characterized by: a saturated atmosphere;

relatively low light intensity (photosynthetic photon flux), averaging 12-70 umol m^{-2} s^{-1}; relatively high and constant temperature (20-28 °C); low rates of gas exchange between the containers and the external atmosphere and high concentrations of carbohydrate and exogenous growth regulators in the medium. Although we are aware of some aspects of the CIP it can hardly be described as well defined. Our knowledge is mostly limited to temperate species and almost exclusively to angiosperms. While in some cases the environmental determinants are known, a direct relationship between certain aspects of the CIP and some component(s) of the culture environment remain obscure.

In vitro shoots and plantlets are invariably diminutive; much smaller than their greenhouse-grown counterparts. Blackberry leaves (Rubus sp.) in culture were only 1-2 % the area of greenhouse-grown control plant leaves [16]. In miniature red raspberry (Rubus idaeus L.) plantlets the proportion of foliar cell and tissue widths to total leaf width were the same in culture as for the large greenhouse-grown control plants [18]. Mature leaves of red raspberry plantlets always had palisade:epidermal cell ratios of 1:1 or 2:1, typical of very young control leaves prior to epidermal cell expansion and palisade cell division [21]. Microcultured Asian white birch (Betula platyphylla var szechuanica (Schneid) Rehd.) was also shown to be small, more from decreased cell division than reduced cell size [58]. The relatively high cytokinin concentration, especially in Stage II [52] media and the low water potential of most media tend to inhibit apical dominance and affect stature. Media that are more dilute or lacking in cytokinins (many Stage III media) promote the development of larger organ size (Donnelly, pers. obs.). In vitro shoots and plantlets have increased percentage water content and reduced dry matter accumulation per unit area compared to greenhouse-grown plants [2, 19]. This is reflected in fragile organs with reduced mechanical support tissue and thin cell walls. Reduced mechanical

support tissue formation occurred in all organs of red raspberry [21]. This could have been nutritionally based but could also have been influenced by the tranquil in vitro environment which inhibited cell wall deposition and sclerenchyma and collenchyma formation [21]. In some species vascular connections were fewer, thinner and poorly structured, as in the petioles of Asian white birch [58], the stems of carnation (Dianthus caryophyllus L.) [45] or the root-shoot interface of adventitious cauliflower (Brassica oleraceae var botrytis) plantlets [30].

The relatively low light levels and saturated internal atmosphere promote leaves in vitro that anatomically resemble both shade leaves [4, 44, 50] and hydrophytic plant leaves [31]. They often have reduced or absent epicuticular or cuticular wax, which can lack the characteristic crystalline structure, or differ in chemical composition from that of control plants [4, 5, 23, 25, 28, 30, 57, 59, 60, 63, 64, 73]. In vitro leaves had a thinner or somewhat collapsed epidermal layer [4, 21, 73, 74] with a clearly defined [13, 58] or an absent or limited [4, 23, 30, 31, 73] palisade layer, sometimes with obconically-shaped palisade cells [13, 18] and a loosely organized spongy mesophyll with an increased percentage air space [4, 13, 18, 23, 73]. Palisade development is related to light levels [22] and is reduced in vitro as the light levels are relatively low [4]. Increasing the light intensity in sweetgum (Liquidambar styraciflua) cultures increased leaf thickness, promoted palisade differentiation and decreased the percentage air space in the mesophyll [44]. In red raspberry, leaves in culture were simple, rather than compound. This may have resulted from incubation at relatively high, constant temperatures (27 °C) [21]. They also had fewer trichomes and an altered distribution of glandular and thick-walled unicellular hairs compared to greenhouse-grown plants. Increased light intensity in culture promoted filiform but not other trichome formation [18].

Stomatal frequency and density was higher [44, 74] or lower [4] in vitro depending on the species, and the stomatal index was not greatly affected [5] or was lower [13] compared to control plants. Stomata on leaves in culture were more circular in shape [50, 51], larger [5, 50, 51, 74] and had larger substomatal cavities [21] than stomata on control leaves. Size of the substomatal cavities has been correlated to the amount of water stress; largest when the relative humidity is highest [48]. Stomatal aperture is usually larger in vitro than on control leaves [2] with guard cells raised above the epidermal layer [16, 44, 73, 74] but can differ in vitro depending on the stage of culture or where the leaf is situated on the shoot. Stomatal aperture gradually decreased in chrysanthemum leaves towards the less mature leaves of the shoot apex [72]. In vitro stomata have slow response times or impaired function [2, 50, 63, 64, 72]; they do not close in response to stimuli such as darkness, abscisic acid application, solutions with high osmolarity (mannitol or sucrose) or when exposed to high levels of carbon dioxide [3, 72, 74, 77]. In chrysanthemum, further opening was possible in CO_2-free air and higher light intensity or through cytokinin exposure and was followed by closure to their original aperture [72]. Interestingly, guard cell protoplasm was seen to react appropriately when leaves from culture were placed in solutions of various osmolarities or containing abscisic acid. So, impaired stomatal function may result from mechanical rather than physiological causes; reduced or altered distribution of cellulose microfibrils in the guard cell walls affecting cell wall elasticity [72, 77]. Guard cell walls of in vitro Prunus cerasus L. were thinner [50, 51] and lacked invaginations of the anticlinal epidermal cell walls next to the inter-guard cell wall ends, present in acclimatized or greenhouse-grown plants [51].

The hydathodes of rosaceous species in vitro were simpler and had fewer water pores with larger apertures and reduced epithem, the tissue that recovers

solutes from the tracheids, compared to greenhouse-grown plants. This may result from the high relative humidity or the low water potential of the medium in vitro compared to greenhouse conditions [14, 15, 17].

In vitro plants rely principally on sugar as a carbon source [6] and CO_2 uptake capability is low [19, 20, 33, 34, 38]. Microcultured birch photosynthesized at one third of control plant levels [58] and red raspberry at about one quarter of control plant levels [19] at saturating light intensities. In vitro shoots and plantlets are mixotrophic in their mode of nutrition; they apparently alternate between carbohydrate use and CO_2 fixation. Carbohydrate use is stimulated by the high concentration of sugar and the presence of growth regulators in the medium and the relatively low light intensity during incubation. Carbon dioxide fixation is stimulated for a short time each day; the CO_2 concentration in the containers is rapidly depleted within about two hours of the start of the photoperiod to at or below the compensation point for the rest of the day [26, 38]. Mixotrophy contributes to the recycling of cellular respiration and photosynthetic products and affects photosynthetic carbon metabolism.

Pigment synthesis and ribulosebisphosphate carboxylase (RubPcase) activity may be impaired in culture; photosynthetic pigment content was low-normal in cultured red raspberry [19] and cauliflower, which also had low RubPcase activity [33, 35]. Some in vitro shoots and plantlets had starch in their chloroplasts [13, 51, 54, 72], while others had little or no starch, as in Leucaena leucocephala (Lam) De Wit. [13] and sweetgum [43, 73]. As sucrose concentration of the medium was augmented, starch concentration in the chloroplasts increased [54]. Little starch was exported from the chloroplasts during the dark period and it tended to accumulate. In vitro leaves exhibited flattened, disorganized chloroplasts, in some cases with swollen thylakoids [43, 54, 73].

Accumulation of starch and disorganization of thylakoid structure was attributed to the relatively low light levels in culture [54] or altered light spectrum resulting from glass containers [43]. However, cauliflower cultures had control levels of photosynthetic electron transport, indicating normal thylakoid structure and function [33].

Propagates are apparently acclimatized to in vitro conditions as their growth is extremely prolific. Furthermore, the in vitro environment affects a wide range of species in a similar way, morphologically and physiologically (Donnelly, pers. obs.). However, a change in a single climatic parameter or medium component may affect one or more of the CIP characteristics, which in turn affects in vitro, and subsequent ex vitro performance.

2.3. Ex Vitro Transplantation

Difficulties in successfully transplanting tissue cultured shoots and plantlets to soil are well documented [6, 25, 66]. They appear to be a direct result of the culture-induced phenotype which reflects adaptation to in vitro conditions but is inappropriate when shoots or plantlets are transferred to the greenhouse or field where the relative humidity tends to be less than 100 %, the ambient light levels are much higher than in culture, there are fluctuating temperatures, the substrate has a much higher water potential and it is necessary to convert rapidly from a mixotrophic to a fully autotrophic mode of nutrition to survive.

Ex vitro plantlets have extreme evapotranspiration rates and may guttate copiously, demonstrating impaired ability to regulate water loss. Excessive evapotranspiration is affected by reduced or nonexistant stomatal control [2, 3, 5, 51, 61, 65, 71, 72, 77], and large cuticular water losses [2, 50] possibly due to poor epicuticular and cuticular wax formation [61] or reduced trichome numbers [18, 60]. The major mechanism of water loss may depend on the

species in question, as some species have large quantities of epicuticular wax in vitro but still have water regulation problems ex vitro [60, 61, 65]. No correlations have been established between ex vitro survival and the physical or morphological characteristics of foliar wax [60, 65]. The ex vitro guttation rate may be affected by the large increase in substrate water potential at transplantation and tends to increase under conditions that augment the transpiration rate [15]. Ex vitro root function is uncertain at the time of transplantation, especially in adventitious propagation systems, and may contribute to water deficit in transplants [1, 6, 30].

To promote ex vitro survival and physiological competence; especially to guard against water stress and encourage autotrophy, a transitional environment is usually supplied for an acclimatization interval, ranging in duration from one to several weeks [3, 4, 6, 23, 34]. In this transitional environment the relative humidity is kept in the range of 70-100 % via tenting, misting or fogging and the light level should not be too much greater than it was in culture. Red raspberry survival was optimal when the light intensity did not initially exceed a three-fold increase over that found in culture [21]. Growth was not limited by light since CO_2 uptake was not different in transplants grown at light intensities two- to three-fold higher than in culture [19]. Gradually, as the plantlets acclimatize, the relative humidity can be decreased and the light levels can be increased towards ambient.

2.4. Transplant Phenotypes Ex Vitro

2.4.1. The Persistent Leaves

Leaves that developed in culture were retained after transplantation for a week to several months prior to senescing [18, 19, 21, 32, 34]. Persistence depended on the plant species and the degree of environmental stress ex vitro.

These persistent leaves increased in size slightly, mainly due to cell elongation [23, 31], and accumulated dry matter under certain conditions [19]. In some cases wax was deposited on the leaf surface after transplantation [23, 30, 50, 64, 71, 75]. Stomatal function (open-closure mechanism) was either improved [51] or was not established in persistent leaves [70]. In most cases stomatal function has been equated with closure [2-4, 18, 50, 51, 61, 65, 74]. However, stomatal closure may only indicate the collapse of the guard cell membranes in response to exposure to low levels of relative humidity [5] and need not indicate the stomatal capacity to reopen.

The role of the persistent leaves remains a controversial and important issue. Photosynthetic capacity appears to vary with plant species in culture and may determine the ex vitro contribution of persistent leaves. Cultured plants are divisible into photosynthetically non-competent and competent species [29]. For example, in the non-competent species group, cultured cauliflower [32] and strawberry [34] were net respirers both in vitro and after transplantation. In these species, leaves that developed in culture deteriorated rapidly after transplantation. These leaves contributed only those nutrients which could be resorbed by the transplant. Such leaves have been referred to as storage organs or pseudo-cotyledonary tissues [34, 69, 71]. Non-competence in strawberry has been attributed to irreversibly reduced levels of RubPcase activity in leaves developed in the presence of sucrose Strawberry plantlets defoliated in the absence of sucrose in the medium were competent [29, 35]. Dieffenbachia (Dieffenbachia picta) [33] as well as potato (Solanum tuberosum) and chrysanthemum (Chrysanthemum morifolium) [29] were photosynthetically competent in vitro. They achieved a positive carbon balance in culture and continued to contribute photosynthetically after transplantation. Leaves of competent species did not deteriorate rapidly after transplantation [29, 33, 69]. Persistent leaves of Asian

white birch [58] and red raspberry [19] seem to fall into the competent group. Red raspberry plantlets photosynthesized at a low level after transplantation. However, persistent leaves shifted to become both net respirers and sinks for photoassimilates formed in the new leaves by one month ex vitro [19]. Retention time of these persistent leaves ranged up to three months ex vitro [18].

2.4.2. The New Leaves

The phenotype of new leaves formed ex vitro varies with the species, the culture and transplant environments and the age of the transplant. New leaves of cauliflower (a non-competent species) that formed the second week after transplantation apparently exhibited greenhouse control levels of CO_2 uptake [30]. However, new leaves of red raspberry (a competent species) were transitional in the sense that weekly flushes of new leaves became progressively larger, eventually with control-type anatomy, functional stomata and improved CO_2 uptake capability [18-21]. Measured five weeks after transplantation leaves formed during the first week had activity levels much higher than in culture, but resembled the cultured leaf phenotype while those formed the fifth week were operating at about half the control CO_2 uptake rates and anatomically resembled greenhouse-grown control plant leaves [20]. Transitional leaves have also been observed in other plant species, both competent and non-competent [13, 23, 50, 73, 74].

The number of transitional leaves produced by a transplant may depend on the number of immature leaf buds formed in culture. The degree of transition of these leaves and how closely they resemble those of control plants is probably a reflection of the stage of development of leaf primordia when the plantlet was transferred from culture and the conflicting stresses imposed on leaf development by both the culture environment and the new ambient

environment [18, 19, 21]. It is likely too, that the retention of any culture-type organs on the transplant influences the physiological status of the rest of the plant [21].

2.5. Acclimatization Strategies for Micropropagated Plants

2.5.1. Acclimatization Ex Vitro

Traditionally the acclimatization environment ex vitro is adjusted to accomodate transplants from culture; gradually weaning them towards ambient relative humidities and light levels. As previously mentioned, 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 the in vitro culture conditions [19, 21]. While Stage III plantlets are generally easier to transfer to soil than Stage II shoots, whenever possible shoots are preferred due to economic considerations [6].

There are inherent limitations in the efficiency of conventional transplantation protocols. For non-competent species the transplanting risks are much greater than for competent species. However, even the competent species can be slow to adjust to lower relative humidity and take time to become photosynthetically efficient.

Much has been written on the optimization of ex vitro transplant environments for tissue cultured shoots and plantlets [6, 7, 10, 75]. Novel approaches to ex vitro acclimatization include CO_2 enrichment without [42] or with supplementary lighting [11]. These reduced the ex vitro acclimatization interval in controlled humidity chambers or greenhouses but failed to eliminate the requirement for habituation to low humidity [11]. Among the most sophisticated ex vitro acclimatization procedures utilizes the "acclimatization unit" [36, 40], an apparatus which emerged from the engineering science of climate-controlled green-

houses. The micro-computer controlled acclimatization unit can determine the relative humidity, temperature, light intensity, CO_2 concentration, air flow rate and even the temperature of the nutrient solution and has the potential to control almost every other feature of the environment. All facets of the environment can be made to change by increments over time; ranging from simulated in vitro conditions at transplantation to that of the greenhouse or the open field weeks later. In the beginning changes are made in small increments which are later increased. Special emphasis is placed on minimizing water stress in the early stages ex vitro. It is not surprising that in such a unit both transplant survival and growth rates are significantly increased. For now, this enviable research tool is beyond the reach of most scientists.

Antitranspirants have not proven useful in promoting ex vitro survival or performance; phytotoxicity and interference with photosynthesis were both cited as possible reasons [62]. Other leaf surface-covering agents such as glycerol, paraffin wax and grease promoted ex vitro survival of several herbaceous species but have not been evaluated over the long term or examined on woody species [55].

2.5.2. Hardening-off In Vitro

Murashige [52] was first to promote hardening of plants during Stage III. This facilitates but does not, of course, preclude acclimatization ex vitro. Murashige recommended the reduction of medium nutrients, the use of auxins for rooting and increased light levels. Three major strategies have emerged that focus on substantially changing the in vitro environment, especially in the later stages of micropropagation, in order to modify the CIP towards improved storage capability, photosynthetic competence or water relations and thus facilitate transplantation.

The first strategy assumes that larger persistent leaves, packed with greater amounts of storage compounds, would contribute more after transplantation. Increasing the concentration of sugar in the medium might maximize the nutrient function of persistent leaves [11, 34]. To some extent this strategy has been discounted as apt to heighten evapotranspiration losses in transplants [11, 34]. However, it seems to hold promise for some plants [46, 54].

The second strategy assumes that autotrophic cultures will have persistent leaves that live longer and would be more photosynthetically productive ex vitro [34]. The objective is to modify the CIP towards autotrophy in culture. To do this, the oxygen concentration can be reduced in the culture environment, which depresses the photorespiration rate [56]. Alternatively, the sugar is reduced or completely eliminated from the medium [33-35, 38] while the photosynthetic photon flux [12, 38, 41] and the carbon dioxide concentration [12, 41] are increased [8]. Increasing the light intensity alone cannot raise the net photosynthetic rate for cultures at their CO_2 compensation point. Such a "photoautotrophic tissue culture system" (PTCS) has the added advantage that microbial contamination is less of a problem when sugar is eliminated from the medium [27]. In this system a gas permeable, clear plastic film is used as a vessel closure [38]. This plastic film improves gas exchange to the cultures; CO_2 enrichment or O_2 reduction; increases the light penetration to the container contents and decreases the relative humidity of the vessels. Strawberry shoots rooted in the PTCS unit had dry weights 1.7-fold greater and photosynthetic rates 4-fold higher than plantlets in the control, Stage III treatment [27]. No special ex vitro care was required for some plants [36, 37] although water stress was still a problem for others [27]. In adopting the PTCS, in vitro culture has been exchanged for a hydroponic system. This has the advantage that contamination by bacteria and fungi are no longer a problem in vitro, extensive

climate control, larger vessels and robotization are possible and the ex vitro acclimatization stage is less of a problem [39]. However, one must accept the concurrent loss of many of the advantages of micropropagation, such as growth rates and miniaturization [8] and assume the problems of hydroponic systems, such as algae control and the requirements for chronic nutrient solution adjustments [27].

The third strategy assumes that plants developed under lower relative humidity will have fewer transpiration and translocation problems ex vitro and persistent leaves that look more like control plant leaves. The objective is to modify the CIP away from the characteristic hydrophytic-type anatomy and promote epicuticular and cuticular wax development, stomatal function and possibly overcome other deficiencies. Lowering the relative humidity in vitro has been done experimentally with varying results using desiccant in or around the culture container, by coating the medium with oily materials, or both [57, 64, 76], by opening culture containers into low relative humidity atmospheres [2], adjusting culture closures to reduce the relative humidity [57], using special closures that facilitate water loss [24] or by cooling container bottoms [67, 68]. Generally the relative humidity could not be lowered to less than 80-85 % without culture injury [57, 76]. A relative humidity of 85 % decreased the multiplication rate of carnation but increased the number of glaucus leaves, the pigment and protein content, decreased the percentage water content and improved ex vitro survival [76]. At 80 % relative humidity, growth rates of cauliflower and chrysanthemum were similar to those of controls grown under 100 % relative humidity but ex vitro transplantation was greatly facilitated by functional stomata and greater epicuticular wax deposition in plantlets rooted at the lower relative humidity [57]. Increasing the sugar or agar concentrations or adding osmotic agents such as polyethylene glycol to the medium will also lower the relative

humidity and in some cases served the same purpose as desiccants [45, 49, 57, 76]. Cooling container bottoms created a temperature gradient of about 5 °C, condensing water vapor on the surface of the medium and lowering the relative humidity at plant height inside the containers [67, 68].

By decreasing the relative humidity in culture containers, both transpiration and translocation systems are presumably improved in cultured plants [7, 8] with associated improvement in mineral ion uptake through the transpiration stream and other benefits [9]. The obvious disadvantage of more extreme relative humidity reduction was to decrease the multiplication rate [57, 75, 76], posing an obvious dilemma [76]. Ziv [75] recommended that relative humidity reduction should be considered in vitro, even at the expense of reduced propagation rates. As the propagation rate of cauliflower and chrysanthemum was not apparently compromised at 80 %, relative humidity reduction was followed by the elimination of sucrose which successfully promoted autotrophy in Stage III cultures of both these plants [57]. This resulted in comparable photosynthetic rates for plantlets rooted at 80 % relative humidity in vitro and seedling plants, and underlined the viability of this approach.

2.6. Conclusions and Prospects

It is premature to advocate any one of the several, not mutually exclusive, acclimatization strategies presented above. Conventional ex vitro acclimatization works for many but not all micropropagated plants. When such protocols are refined, they succeed in shortening the establishment interval and promoting survival, but do not usually eliminate the necessity for low humidity habituation. The costs of facilities dedicated to ex vitro acclimatization can also be a limiting factor [29]. In order to deemphasize ex vitro acclimatization and reduce associated costs it may be necessary to precede this stage by in vitro

treatments. In vitro hardening-off procedures may be appropriate for some plant species, but are apparently not advantageous for all. Modification to the CIP to promote improved photosynthetic competence and water regulation in shoots or plantlets at the later stages of micropropagation is deceptively simple. As more documentation results from the implementation of these varied acclimatization strategies, alone or in combination, it will be possible to make choices more accurately. These would probably be made on a plant by plant basis with attention to economic considerations.

Tissue culturists are in an ideal position to evaluate the phenotypic plasticity of plants and to sort out the environmental determinants of plant phenotype, since they have access to large numbers of genetically identical plants grown under climate- controlled conditions. In this way we can learn more about the adaptational responses of all plants to specific environmental cues and climatic changes, and can also meet an urgent industry objective, to successfully accomodate or manipulate the culture-induced phenotype in order to promote survival and performance ex vitro.

2.7. Summary

Ex vitro acclimatization of micropropagated plants can be difficult, costly and may limit commercial micropropagation. Solving ex vitro acclimatization problems is contingent on an improved understanding of the unique effects of the in vitro and ex vitro environments on plant phenotype.

Micropropagated plants possess a unique culture-induced phenotype (CIP), reflecting acclimatization to in vitro conditions of: saturated environment; low light intensity; high and constant temperature; low rate of gas exchange between the containers and the external atmosphere and high concentration of carbohydrates and growth regulators in the medium. Many species are affected

in a similar way, both morphologically and physiologically, in vitro. Plantlets are tiny with increased percentage water content and have fragile organs with reduced mechanical support tissue, thin cell walls and, in some species, fewer vascular connections. Leaves in vitro resemble those of shade and hydrophytic plants; with reduced or altered epicuticular and cuticular wax, thinner epidermal layers and loosely organized mesophyll with increased percentage air space compared to control leaves. Stomata of cultured plants may vary in density, shape, size and function compared to those of control plants. Impaired stomatal function of persistent leaves appears to result from mechanical and not physiological causes. In vitro plants are mixotrophic; alternating between carbohydrate use and CO_2 fixation. Pigment synthesis and ribulosebisphosphate carboxylase activity may be affected in culture.

The CIP is inappropriate when plants are transferred from culture to greenhouse or field conditions. Ex vitro transplants have extreme evapotranspiration rates due to reduced stomatal control, large cuticular water loss and, in some species, reduced trichome numbers. Water regulatory problems may be affected by ex vitro guttation and in certain adventitious propagates impaired root function. Some species are photosynthetically non-competent both in culture and after transplantation while others are competent. In non-competent species persistent leaves deteriorate rapidly after transplantation, behaving like storage organs, while in competent species they do not. New leaves of transplants are transitional; varying phenotypically from culture- to control-type with successive flushes of new leaves. The degree of transition is affected by the stage of development of leaf primordia before transfer from culture and conflicting stresses on leaf development imposed by the culture and transplantation environments.

Conventional ex vitro acclimatization involves a gradual weaning of transplants from culture conditions towards ambient relative humidity and light

levels. This is more difficult for unrooted shoots and non-competent species than for plantlets or competent species. Modern approaches involve supplemental CO_2 with or without supplemental lighting, and use of a completely climate-controlled "acclimatization unit". Hardening-off in vitro plants facilitates but does not preclude acclimatization ex vitro. Three major strategies for in vitro hardening have focused on changing the culture environment to modify the CIP towards improved storage capability, photosynthetic competence or water relations. Increasing the sugar concentration of the medium to maximize the storage capability of persistent leaves holds promise for some species. The promotion of autotrophy via CO_2 enrichment or O_2 reduction, reduced levels of sugar and increased light intensity is promising for some plants, although water stress remains a problem at transplantation. Lowering the relative humidity to 80-85 % via several methods did little to compromise the multiplication rate for certain species and facilitated transplantation. Elimination of sugar from the medium, in conjunction with reduced relative humidity, promoted autotrophy for both a competent and a non-competent species.

In vitro hardening-off procedures are relatively new and have not yet been widely evaluated. It is premature to advocate any one of the several, not mutually exclusive, acclimatization strategies outlined above. Tissue culturists have access to large numbers of clonal plants grown under defined environmental conditions. They are in an ideal position to define the environmental determinants of plant phenotype and characterize plant phenotypic plasticity.

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Clearing and staining leaves is useful for observing anatomical details including stomatal index (Arnott, 1959, O'Brien and McCully, 1981). The method's main advantage over other techniques is the short preparation time and the facility with which large sections of a leaf can be observed at one time. A variety of methods were recommended in the literature, many requiring several days to complete with the results being, at times, inadequate (Rodin and Davis, 1967, Shobe and Lersten, 1967). An attempt was made to develop a clearing and staining method rapid enough to enable large numbers of samples to be cleared and stained within 1 day. It was also hoped that the method developed could be used for a range of different plant species with varying leaf morphologies as well as for fragile tissue-cultured material.

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A Rapid Clearing and Staining Method for Tissue-cultured Plantlets and Greenhouse-grown Leaves

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Abstract. **A simple, rapid clearing and staining method was developed using intact in vitro blackberry (*Rubus* spp. 'Silvan') and strawberry (*Fragaria ×ananassa* Duch. 'Totem') plantlets and sections of greenhouse-grown 'Silvan' and 'Totem' leaves. The clearing method involved three steps: 1) autoclaving in 80% ethanol to remove the chlorophyll, which took 15 to 20 min for plantlets and 25 and 35 min for greenhouse-grown 'Silvan' and 'Totem', respectively; 2) dissolution of the protoplasm using 5% NaOH at 80°C, which took 20 min for plantlets and 35 and 40 min for greenhouse-grown 'Silvan' and 'Totem' respectively; 3) post-alkali treatment with 75% bleach (4.5% NaClO). For tissue-cultured plantlets this took 5 to 10 min at room temperature, but greenhouse-grown material required 35 to 40 min at 55°. Aqueous safranin (10 mg liter^{-1}) was used for staining. This method gave consistently good results and required a maximum of 2 hr for completion.**

When plant material is cleared, there is a selective dissolving of certain cell components, based on their chemical and/or physical properties, for the purpose of observing others. Organelles and nonlignified tissues are the first to be removed and dense, resistant lignified elements usually persist throughout the clearing process (7).

Leaf clearing has been used for morphological identification and anatomical observations, as in the examination of surface structures (stomates and hairs) or xylem (1, 7). Cleared preparations may supplement microtome cuttings by giving a better idea of spatial relationships between leaf tissues (1). Despite its usefulness, tissue clearing is a technique that has been largely underemployed. Clearing and staining methods have been gathered and summarized (4, 7). Many clearing methods are excessively time-consuming, requiring several days (9, 10), or have many steps with several different, sometimes toxic (i.e., pyridine), chemicals (5), while the final results are inadequate.

Our intention was to develop a rapid, simple, and reliable clearing method for tissue-cultured plantlets and greenhouse-grown leaves of raspberry and strawberry. To this end we sought to improve the most comprehensive three-step clearing processes. These involve: 1) pretreatment, 2) dissolution of protoplasm, and 3) post-alkali treatment. Pretreatment involves removal of chlorophyll, usually with 70% or 80% ethanol, leaving a white, opaque leaf. The protoplasm is subsequently dissolved, rendering the leaf transparent. Protoplasm is removed using a strong alkali such as NaOH, which reacts with phenolic compounds, turning the leaf a uniform brown. This brown coloring is removed by bleaching the leaf in sodium hypochlorite (NaClO) (7). In vitro plantlets of 'Silvan' blackberry and 'Totem' strawberry were representative of very young leaves or fragile material since plantlet leaves had almost no cuticle, very thin cell walls, and few cell layers. Greenhouse-grown plant leaves have more cuticle, thicker cell walls, and larger, thicker leaves than tissue-cultured plantlets (2, 3) Greenhouse-grown 'Silvan' leaves have less cuticle than 'Totem' strawberry and are slightly thinner.

Table 1 Summary of clearing method for intact tissue-cultured plantlets and greenhouse-grown plant leaves of 'Silvan' blackberry and 'Totem' strawberry.

Cultivar	Treatment: Pretreatment[z] (min)	Alkali[y] (min)	Post-alkali[x] (min)
	Tissue-cultured		
Silvan	10–20	20	5–10 (RT)
Totem	10–20	20	5–10 (RT)
	Greenhouse-grown		
Silvan	25	35	35 (55°C)
Totem	35	40	40 (55°C)

[z]Pretreatment involved autoclaving in 80% ethanol.
[y]Alkali treatment involved exposure to 5% NaOH at 80°C.
[x]Post-alkali treatment involved exposure to 75% bleach at room temperature (RT) or at 55°C.

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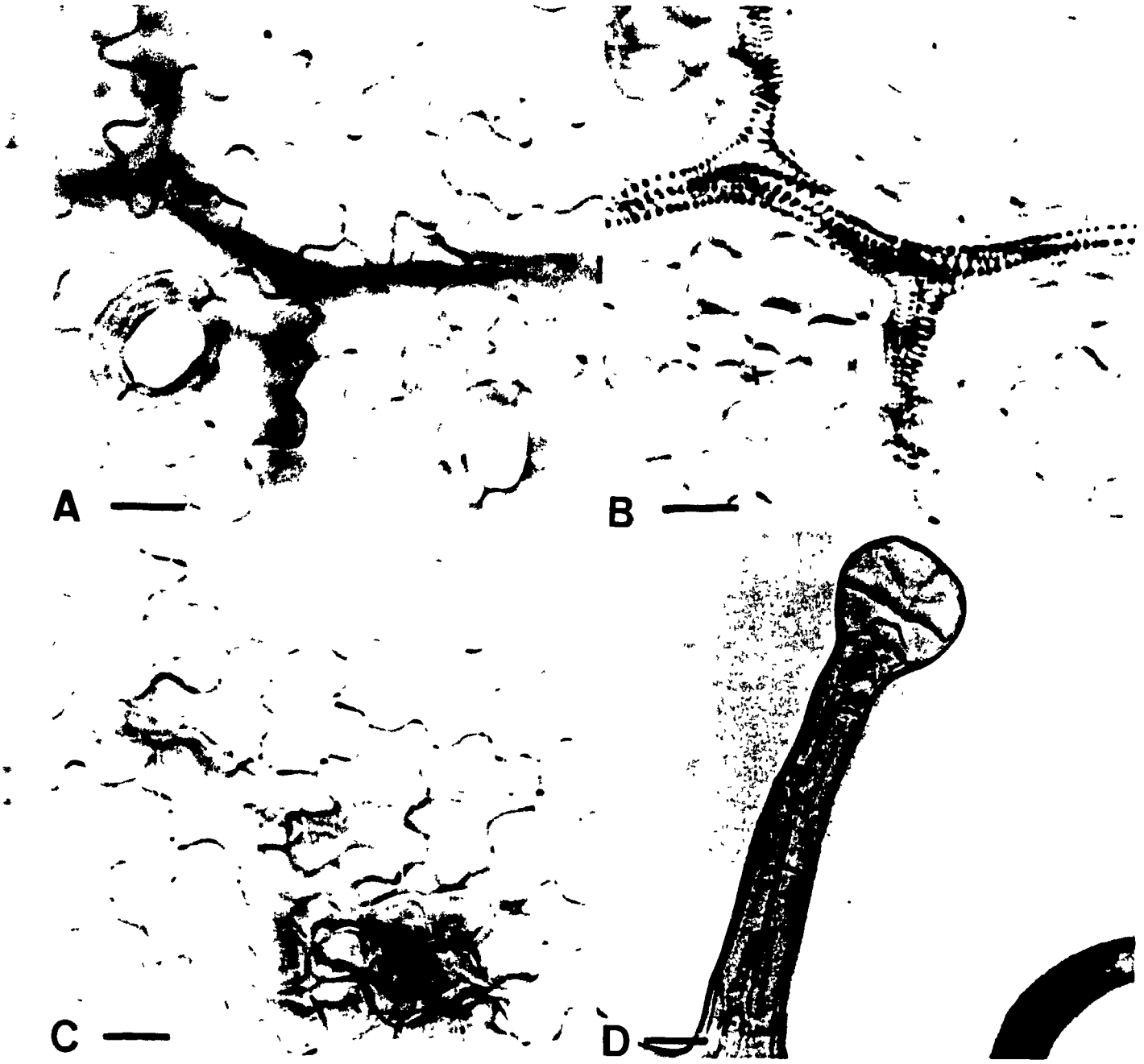

Fig. 1 Photomicroscopic view of an in vitro 'Silvan' blackberry leaflet, focussing down from abaxial to adaxial surface (**A**) Abaxial leaf surface (**B**) Internal view of vascular tissue and palisade layer (**C**) Adaxial surface. (**D**) Glandular trichome (colleter) Micrometer bar = 50 μm (**A–C**), 100 μm (**D**).

In vitro plantlets were grown on wicks in liquid Murashige and Skoog medium (6) The plantlets were grown under 38 μmol s^{-1}·m^{-2} cool-white fluorescent lights with a 16-hr photoperiod. In vitro plantlets were cleared intact. Greenhouse-grown plants were kept at ambient temperatures with a 16-hr photoperiod. Leaves were cut into 0.44-cm^2 disks using a paper punch or into 1- or 2-cm^2 sections. Disks were taken at random and in some cases included the midrib area. Disks were used solely for initial pretreatment experiments where precise area was essential. Square sections were used in subsequent pretreatment, protoplasm dissolution, and post-alkali stage experiments since they provided a larger area for treatment evaluation. The square sections were cut just below the tip of a leaf and always included a midrib section.

Specimens were rinsed three to five times in distilled water between each of the three clearing stages. A small strainer was used to prevent specimen loss from the bottles. Safranin was used to stain the specimens after the post-alkali stage and water soak.

Pretreatment stage. To determine the most effective concentration of ethanol for removing chlorophyll, 10 blackberry leaf disks from greenhouse-grown plants were placed into 50-ml specimen bottles in 40 ml of 50%, 60%, 70%, 80%, 90%, or 100% ethanol. The containers were autoclaved at 103.4 × 10^3 Pa and 120°C for 15 min, removed from the autoclave, and the caps closed tightly to prevent evaporation of ethanol. This experiment was repeated three times The ethanol–chlorophyll solutions were filtered and immediately analyzed spectrophotometrically at the chlorophyll absorbance peak of 652 nm

When using the square sections required for later steps, the thoroughness of chlorophyll removal was visually assessed by specimen color. Successful pretreatment resulted in the leaf section turning opaque Residual green indicated that all the chlorophyll had yet to be removed whereas browning meant that autoclaving was excessive or an insufficient initial volume of ethanol was used, causing either a phenolic reaction or burning

Dissolution of protoplasm Pretreated leaf sections were used to evaluate the most effective NaOH concentration, temperature, and

exposure time for complete protoplasm removal. NaOH was used at concentrations of 1%, 2%, 5%, and 10% at 20°, 55°, and 80°C for periods ranging from 15 min to 1 hr, in 5-min increments. The effect of autoclaving (8) was tested. The time for complete protoplasm removal could only be determined by microscopic observation after the phenolic compounds had been bleached in the post-alkali stage. The protoplasm dissolution stage was deemed complete when microscopic observation showed the spongy mesophyll and the palisade cells to be free of organelles.

Post-alkali stage. The optimum pretreatment and protoplasm-dissolution stage treatments were employed using new specimens. The most effective concentration of household bleach (6% NaClO) for removing the brown phenolic oxidates was determined. Concentrations of 25%, 50%, 75%, and 100% bleach (1.5%, 3%, 4.5%, and 6% NaClO) were employed at 20°, 55°, 60°, 65°, and 75°C until the samples were clear. Effectiveness of the post-alkali stage was based on the time needed to bleach specimens from brown to transparent and upon the mechanical strength of samples after treatment. A specimen was deemed clear once every spot of brown had been bleached.

Cleared specimens were stained in aqueous safranin (10 mg·liter^{-1}) and mounted in glycerol for semi-permanent mounts. Permanent mounts required dehydrating cleared, unstained material through a series of ethanol concentrations, staining in 95% or 100% ethanol, rehydrating into xylene, and mounting in Permount.

Pretreatment stage. Spectrophotometric analysis of chlorophyll extracted from 'Silvan' leaf disks showed that 80% ethanol, within the 15-min autoclaving interval, extracted the most chlorophyll. Ethanol concentrations below 80% removed less chlorophyll during this interval. Ethanol at 50% to 70% eventually dissolved all of the chlorophyll, but required longer autoclave times and a much larger volume of ethanol since evaporation was greater over the extended autoclave intervals. Ethanol concentrations above 80% removed chlorophyll less effectively. Chlorophyll removal from intact plantlets grown in vitro took 15 to 20 min in 80% ethanol in the autoclave. For greenhouse-grown leaves, chlorophyll removal time was 25 min for 'Silvan' and 35 min for 'Totem'. The time differences for chlorophyll extraction were probably due to different amounts of epicuticular and cuticular wax (7).

Dissolution of protoplasm. The most rapid treatment for dissolving the protoplasm was 5% NaOH at 80°C for various periods of time depending on the type of tissue. Intact plantlets grown in vitro needed 20 min; leaf squares of mature greenhouse-grown 'Silvan' and 'Totem' leaves needed 35 min and 40 min, respectively. NaOH at 10% caused tissue disintegration, as did autoclaving in NaOH for most samples.

Post-alkali stage. Immersion in 75% bleach at room temperature for 5 to 10 min was the preferred post-alkali treatment for in vitro plantlets. Greenhouse-grown material in 75% bleach at 55°C became transparent in between 35 to 40 min. Bleach concentrations below 75% took longer to achieve the same results, but 100% bleach or temperatures at or above 60° macerated the tissues. The midrib and petiole, due to their thickness, were the last areas from which phenolic compounds were totally bleached.

It was essential to remove the bleach completely by soaking for a few minutes in distilled water before staining with safranin because of the destructive interaction of safranin and sodium molecules. In vitro plantlets stained adequately in only 10 to 30 sec whereas greenhouse-grown specimens took 1 to 2 min if superficial tissues, such as hairs or guard cells, were of interest or 5 to 7 min if internal tissues, such as xylem, were to be examined.

Since in vitro plantlets were cleared intact, microscopic observation of the external and internal structures of the root, stem, petiole, and leaves was possible. All the cell layers from adaxial to abaxial leaf surfaces could be microscopically observed (Fig. 1 A, B, and C). When mounted abaxial side up, the adaxial epidermal layer was not always quite as clear as the upper surface due to foliar width, but it was usually not difficult to discern cell patterns or stomata. Differences in cell shapes and patterns between palisade and spongy mesophyll layers were apparent.

In the case of greenhouse-grown leaves, sections including the midrib were more likely to exceed 2 to 3 mm in width. Mounted adaxial side up, the lower epidermal layer was usually out of focus due to the thick epidermal cells, the many cell layers and the difficulty in keeping the coverslip adhered to the specimen. For optimum examination of both leaf surfaces, specimens were halved and mounted both adaxial and abaxial side up, examined and subsequently turned over, or the midrib was removed.

In summary, the clearing and staining method outlined in Table 1, with minor variations, gave rapid (maximum time of 2 hr) and excellent results for the plants examined. This clearing procedure was particularly successful with in vitro plantlets for which organs and cell layers were made clearly visible. Due to its speed and reproducibility, this procedure may prove to be a useful laboratory tool to facilitate stomatal counts and measurements. It should be especially useful as a method for showing the three-dimensional relationships of cell layers: vascular tissues, crystal deposits, hydathodes, trichomes (Fig. 1D), and other foliar structures. We have found the method useful in obtaining a general view of an area that is to be examined later using microtome cuttings.

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Having developed a satisfactory clearing and staining method for observing tissue-cultured and greenhouse-grown plant material, acclimatization experiments were initiated in order to 1) determine the effects of both increased agar concentrations (6, 9 and 12 g/l) in the culture medium and in vitro rooting on ex vitro survival, growth and stomatal index of micropropagated 'Silvan' blackberry shoots and 2) determine the effect of high relative humidity and low light intensity on the stomatal function of leaves from a) ex vitro plantlets grown on full and 1/4 strength modified MS (1962) basal medium and b) greenhouse-grown 'Silvan' plants.

4. Acclimatizaion of micropropagated 'Silvan' blackberry

4.1. Introduction

Ex vitro acclimatization can be one of the most difficult stages of the micropropagation process. This is due to the impaired ability of transplants to control water loss as a result of poorly formed epicuticular and cuticular wax layers (Conner and Conner, 1984, Fabbri et al., 1986, Sutter and Langhans, 1982, Sutter 1984, 1985), non-functional, open stomata (Brainerd and Fuchigami, 1981, Donnelly et al., 1986, Short et al., 1987, Wardle and Short, 1983) and, in some cases, poorly formed root systems (Grout and Aston, 1977).

Generally, plants grown under culture conditions have reduced foliar epicuticular and cuticular wax. In vitro-formed foliar wax may be different in arrangement and chemical composition compared with that of greenhouse-grown plants (Sutter, 1984, 1985). Reducing the relative humidity level in vitro from 100 % to 35 % by using a variety of closures for the culture containers increased epicuticular wax formation in cauliflower (Brassica oleracea var botyris) and chrysanthemum (Chrysanthemum morifolium Ram.) (Short et al., 1987). Increasing the agar concentration from the usual 6-8 g/l to 15 g/l resulted in decreased relative humidity in the culture containers; from 98 % to 89 %. Subculture to medium with an agar concentration of 15 g/l 2 weeks prior to transplantation promoted glaucous leaf production in carnation (Dianthus caryophyllus) plantlets and improved transplant survival from 11 % to 72 % (Ziv et al. 1983). Similarly, increasing the agar concentration in the rooting medium from 6 g/l to 14 g/l increased the ex vitro survival of micropropagated cherry (Prunus cerasus) rootstock from 13 % to 45.3 % (Marin and Gella, 1987).

Stomata formed on most in vitro plant leaves did not close in response to stimuli such as darkness, abscisic acid, solutions of high osmolarity (mannitol

or sucrose) or exposure to high levels of CO_2 (Brainerd and Fuchigami, 1982, Wardle and Short, 1983, Wetzstein and Sommer, 1982, Ziv et al., 1987). Reducing the relative humidity from 95-100 % to 30-40 % in vitro decreased the time necessary for stomata to close in apple (Malus domestica (Borkh.) (Brainerd and Fuchigami, 1981). Since transpiration losses occur through the open stomatal pores, researchers have investigated stomatal frequency or stomatal index, which corrects for differences in leaf cell size, in culture and during ex vitro acclimatization and compared it with that of greenhouse-grown control plant leaves. Stomatal index (S.I.), described by the formula: S/(S + E) x 100, where S = number of stomata and E = number of epidermal cells, was unaffected by micropropagation in both potato (Solanum laciniatum) (Conner and Conner, 1984) and rose (Rosa multiflora L.) (Queralt, 1988). The S.I. of Leucaena leucocephala (Lam) De Wit. was lower on leaves from micropropagated shoots at the end of the multiplication stage (7.21) than on leaves from the original, mature donor plant (12.02) (Dhawan and Bhojwani, 1987). However, the S.I. of each successive new leaf following ex vitro transplantation has never been documented.

Little attention has been paid to the fate of the persistent leaves from culture during the ex vitro acclimatization period despite the fact that these make up most of the biomass present at transplantation. It is of interest to know whether these leaves contribute to new growth, through photosynthesis or nutrient transfer during senescence. or whether they act as sinks (net respirers) using photosynthates which could otherwise enhance new leaf or root growth. Carbon fixation of persistent leaves of strawberry (Fragaria x ananassa Duch) was insufficient in culture for autotrophic growth and did not increase during the ex vitro acclimatization period. Persistent leaves of strawberry senesced and dropped off the transplant almost immediately (Desjardins et al., 1988) to 4

weeks after transplantation (Grout and Millam, 1985). Grout and Millam concluded that strawberry transplants must be dependant upon stored materials to provide a carbon source for growth and development immediately following transplantation. Wardle et al. (1983) showed that persistent chrysanthemum (Chrysanthemum morifolium) leaves acted as storage organs until they senesced; contributing minerals to the new organs developed during ex vitro acclimatization. Carbon fixation in persistent leaves of red raspberry (Rubus ideaus) was about one quarter that of control plant levels. After transplantation these leaves continued to photosynthesize at low levels. However, they became net respirers and sinks for photoassimilates formed in the new leaves after 1 month ex vitro (Donnelly and Vidaver, 1984b). The persistent leaves of red raspberry did not deteriorate rapidly after transplantation and some were retained on the transplants for up to 3 months. The persistent leaves of 'Silvan' blackberry were similar to those of red raspberry in their retention ex vitro.

There is disagreement in the literature as to the benefits derived from in vitro rooting. For some adventitously propagated plant species, in vitro-formed roots may not be completely functional after transplantation, possibly because of incomplete vascular development between the roots and shoots, contributing to excessive desiccation of transplants on exposure to low relative humidity levels. Vascular connections were narrow and ill-formed in adventitiously cultured cauliflower (Brassica oleracea var. botrytis) restricting acropetal water transfer (Grout and Aston, 1977). In vitro roots of Gladiolus formed in 0.8 % agar were abnormally thin, sparse, and lacked root hairs (Logan and Zettler, 1985). In vitro roots of Acacia koa had no root hairs when grown on agar-solidified medium and frequently lacked a fully developed vascular system (Skolmen and Mapes, 1978). In many plant species there is a delay in the initial growth of in vitro rooted plantlets after transplantation. This too has been attributed

to the lack of a functional in vitro root system at transplantation (Debergh and Maene, 1981, Pierik, 1988). However, initial growth comparisons of shoots and plantlets after transplantation were not reported. In some plant species, roots formed in vitro died after 2 weeks of growth ex vitro and were replaced by new ones (Debergh and Maene, 1981). It is also possible that some in vitro roots are damaged during the planting process, increasing the risk of root and/ or stem disease (Debergh and Maene, 1981).

Rooting of shoots in vitro is labour intensive and therefore costly due to the requirement for individual handling of each shoot. For this reason it may not be economically advantageous to root in vitro, whether the roots are functional or not (Debergh and Maene, 1981). However, axillary shoot propagated in vitro-rooted loganberry (Rubus loganobaccus Bailey) plantlets were hardier after one month ex vitro compared with shoots that were rooted ex vitro (Donnelly et al., 1980). Most commercial micropropagation facilities or companies practice in vitro rooting (Debergh, 1988a). The reason for this is unclear; it is postulated that prejudice against unrooted shoots is due to the habitual use of rooted material for successful transplanting of almost all seedlings and vegetatively propagated shoots.

In vitro acclimatization strategies for raspberry have included culturing shoots on media with reduced (20 %) concentrations of modified MS (1962) macro-elements for 2 weeks prior to transfer (Welander, 1985). This was reported to increase in vitro rooting from 33 % to 100 % and promote survival (from 63 % to 100 %) 1 month after transplantation. Half-strength modified MS (1962) was recommended for in vitro rooting of apple (Malus domestica L.) (Zimmerman et al., 1987). Another in vitro acclimatization strategy is the reduction or elimination of sucrose in the medium. This has been shown to promote photoautotrophy in cultured plants (Grout and Price, 1987, Kozai, 1988a).

The effect of reduced macro and micro nutrients or medium sugar upon certain aspects of plant physiology, such as stomatal function, has not been investigated. The objectives of the following experiments were to:

1) Determine the effect of increased medium agar concentration, from the control level of 6 g/l to 9 and 12 g/l, on survival and growth performance (transplant height, leaf number, longest leaf length) of 'Silvan' shoots and plantlets during the first 3 weeks ex vitro.

2) Determine the relationship between the initial (persistent) leaf number or initial fresh weight with final fresh weight 3 weeks after transplantation.

3) Determine if the stomatal index differed between leaf populations (persistent, new leaves formed during week 1, week 2 and week 3) and if it was affected by different medium agar concentrations or in vitro rooting during the first 3 weeks ex vitro.

4) Find a suitable method to test the open/closure mechanism of 'Silvan' blackberry stomata on epidermal peels.

5) Determine the period of time required after transplantation for stomata to exhibit the capacity to open and close and assess this function in persistent and new leaf populations.

6) Determine the effect of high relative humidity (> 95%) and low light levels (82 $\mu mol\ s^{-1}\ m^{-2}$) on stomatal function of greenhouse-grown 'Silvan' leaves and ex vitro 'Silvan' plantlet leaves from full and 1/4 strength modified MS (1962) basal medium.

4.2. Materials and Methods

4.2.1. Source Plants

'Silvan' blackberry (Rubus sp.) plants developed from 30 cm long stem cuttings were placed in a mist chamber for 2 weeks. The rooted-cuttings were then repotted into 22.5 cm standard pots and grown for 6 weeks in the greenhouse at ambient temperatures (24 ± 5 °C) under natural light supplemented with cool-white flourescent light, to total 100 µmol s^{-1} m^{-2} for 16 hr per day. These source plants were grown in Promix^{R} supplemented with 30 g dolomitic limestone and 150 g osmocote (14:14:14) per 30 cm^3.

For tissue culture purposes cuttings (10-30 cm long containing 2-3 nodes) were taken from the source plants and the leaves removed. The cuttings were washed in distilled water for 10 min and then agitated for 20 min in 10 % bleach (0.6 % NaClO). Surface sterilized cuttings were rinsed in sterile distilled water for 5-10 minutes prior to aseptic shoot-tip excision. In vitro shoots and plantlets were derived from 1-2 mm long shoot-tip explants initiated and grown in 50 ml test tubes containing filter paper wicks and 15 ml of liquid modified Murashige and Skoog (1962)(MS) basal medium with additions (Appendix I). After 2 subcultures, at 4 week intervals, shoots were transferred from test tubes to 400 ml polypropylene containers (10-15 shoots in each) on 50 ml of the same medium (multiplication) solidified with 6 g/l Difco-Bacto agar. The shoots were grown under 57 µmol s^{-1} m^{-2} cool white fluorescent light with a 16-hr photoperiod at a constant temperature of 25 °C.

4.2.2. Transplantation

Transplantation from culture was done into Promix[R] supplemented as previously described. Transplants were watered in with 5 g/l of 10:52:10. A fungicide mixture consisting of 0.5 g/L Truban[R] and 0.5 g/l Benlate[R] was misted onto the transplants the second day after transplantation. Transparent plastic lids were sealed to the flats with tape in order to maintain the relative humidity at 100%. Transplants were hand-misted daily for 2 weeks with a dilute solution of 10:52:10 (3 g/l) and subsequently with a dilute solution of 20:20:20 (3 g/l).

4.2.3. The effect of medium agar concentration and in vitro rooting on 'Silvan' following transplantation

Experiment 1

After 8 subcultures at 4-6 week intervals, 30 shoots having 2-4 leaves each were transferred to multiplication medium or a similar rooting medium (Appendix I) each with 3 levels of agar (6 g/l, 9 g/l and 12 g/l) for a total of 6 treatments (Table 2).

Table 2. Summary of medium type (M=multiplication medium, R=rooting medium) and agar concentration in the six treatments.

Treatment	Medium	Agar (g/l)
1	M	6
2	R	6
3	M	9
4	R	9
5	M	12
6	R	12

Twenty shoots or plantlets per treatment, each with 7-8 leaves and 0.8-1.2 cm tall were placed in randomized complete block groupings of 10 and replicated once in 96-plug plastic germination flats. Transplants from the lowest (control) medium agar concentration (6 g/l) had in vitro roots measuring 2-5 cm. In vitro root length was generally shorter with increasing medium agar concentrations: 1-3 cm (9 g/l) and 1-2 cm (12 g/l). After 3 weeks the flat covers were gradually removed over a period of 3 days. Transplants in the Conviron E-15 growth chamber received 127 µmol s^{-1} m^{-2} cool-white fluorescent light with a 16-hr photoperiod and a constant temperature of 25 ± 2 °C. Once the flat covers were removed, transplants were exposed to a relative humidity of 30-40 %. Leaves formed in culture (persistent) were tagged on the petiole with a small drop of white Liquid Paper® at the time of transplantation and thereafter new leaves were tagged on a weekly basis using different colours of Liquid Paper.

Transplant height was measured from the soil line at transplantation and from the edge of the flat to the base of the newest leaf from week 1 to week 5. Height, total number of leaves (both persistent from culture and new) and the length of the longest leaf were recorded for all transplants 1, 2 and 3 weeks after transplantation. Statistical analysis was done separately for each week. Three randomly chosen transplants per treatment were cleared (Chapter 3) each week to determine the foliar stomatal index (S.I.). The S.I. was calculated from photomicrographs of 60-100 epidermal cells including 15-20 stomata per field of view. Two randomly located fields of view from each leaf were photographed. The total number of stomata counted for each leaf type (persistent, week 1, week 2, week 3 and week 5) at each weekly time interval ranged from 40 to 200 (2-10 leaves). The S.I. of greenhouse-grown plant leaves was determined by clearing two leaves of five source plants

(N = 10) and taking photomicrographs of two randomly chosen areas of the leaves. The total number of stomata photographed was 110.

Arcsin transformations of S.I. data were performed on PC-SAS. Differences between means at the 0.05 level of significance were determined with the Duncan's Multiple Range test using the same program.

Experiment 2

After 12 subcultures at 4-6 week intervals, 60 shoots having 2-4 leaves each were transferred to multiplication or rooting medium as in Experiment 1. At the end of 3 weeks 10 shoots/plantlets from multiplication and rooting media with varying agar concentrations (Table 2) were weighed and their leaves counted, then transplanted in a completely randomized design to 36-plug plastic germination flats. The flat covers were closed tightly for the first 2 weeks and then completely removed. Transplants were grown in a Conviron PGW36 growth chamber under 127 μmol s^{-1} m^{-2} cool white fluorescent light with a 16-hr photoperiod at a constant temperature of 25 °C and a relative humidity level of 80 %. All the plants were harvested to determine final fresh weight. Initial fresh weight originally included the root mass (for plantlets). When harvesting for final fresh weight it was found that, in some cases, there was a large amount of callus at the base of the stem. This was removed to allow a better basis for comparison between treatments. Final fresh weight thus included the above-ground biomass (stem and leaves) with any callus removed.

Statistical analysis of all data was done using PC-SAS. Differences between means were tested using the Duncan's Multiple Range test and regressions were done using General Linear Model tests. Correlations between initial fresh weight and initial leaf number to final fresh weight were tested for fit to linear, quadratic and cubic equations at the 0.05 level of significance.

4.2.4. Preliminary tests on stomatal function

Leaves of greenhouse-grown 'Silvan' were covered in aluminium foil 2 hr before removal. Examination of epidermal peels from these severed leaves showed that most stomata were closed. The peels were then floated on a phosphate buffer (10^{-2} M K_2HPO_4 brought to a pH of 6.9 with 10^{-2} M KH_2PO_4) (Willmer and Mansfield, 1969) and placed under a 120 µmol s^{-1} m^{-2} sodium light. The leaf peels were observed under a light microscope every 5 min for 35 min and stomatal apertures were measured directly using an eye-piece micrometer. After 30 min the majority of stomata were open to some degree.

To determine the most useful test for stomatal function, greenhouse-grown 'Silvan' epidermal leaf peels on phosphate buffer were exposed to a) ABA (2:1 10^{-2} M ABA: phosphate buffer) (Martin et al., 1983), b) light/dark periods or c) 1 M NaCl treatments. All tests were replicated twice.

Five epidermal peels were:

a) floated on the ABA solution while under the sodium light and observed at 5 min intervals to determine the time necessary for stomatal closure. Once the stomata had closed the epidermal peels were refloated on the phosphate buffer under the light and examined every 5 min for stomatal reopening.

b) floated on the phosphate buffer in foil-covered petri dishes and examined every 5 min to determine the time necessary for stomatal closure. Once stomata had closed, the foil was removed and the petri dishes were placed under the light. The epidermal peels were examined every 5 min to observe when stomatal reopening occurred.

c) floated one at a time on the phosphate buffer on a glass microscope slide and a 1M NaCl solution was drawn over them. This was done by using filter paper to absorb the buffer solution from one side of the cover slip and by adding the NaCl solution via pipette from the other side. Once stomatal

closure was observed through the microscope the 1M NaCl solution was replaced by the buffer by using filter paper to absorb the NaCl solution from one side of the cover slip and by adding the buffer solution via pipette from the other end until stomatal opening was observed.

4.2.5. Evaluation of ex vitro stomatal function of 'Silvan' plantlets

Experiment 2

Epidermal peels were always taken from between the second and third horizontal vein at the base of the leaf. In the 2 and 4 week tests, two epidermal peels were harvested from each of 2-3 plants/treatment. In the 3 week test two epidermal peels were harvested from every leaf and 5-6 plants/ treatment were sampled.

Stomatal function was determined using the 1M NaCl test as described in the previous section. Each field of view/epidermal peel contained between 10-20 stomata. Twenty stomata were considered adequately representative of the total leaf surface (Meidner, 1981). The total number of stomata measured per leaf type (leaves which had developed during the first, second and third week) was 40-100 for the 2 and 4 week tests and 150-200 for the 3 week test. Stomatal apertures were measured directly under the microscope using an eye-piece micrometer in the 2 and 4 week tests and in the 3 week test photomicrographs of the stomata were taken before and after application of the 1M NaCl solution. Stomatal apertures were subsequently measured in the dark room by projecting the magnified images of specimen negatives (and a micrometer scale) directly from the negative enlarger onto the bench top.

4.2.6. The effects of high relative humidity and low light intensity on ex vitro stomatal function of 'Silvan' plantlets grown on full and 1/4 strength rooting medium

Experiment 3

Twenty shoots with 2-4 leaves each were transferred from multiplication medium to both full strength and 1/4 strength rooting medium (see Appendix I). Three weeks later the 20 plantlets each from full and 1/4 strength rooting media were randomly planted into two 36-plug plastic germination flats and placed in a dew chamber. The dew chamber had a light intensity of 82.5 µmol s^{-1} m^{-2} cool-white fluorescent light, a 16 hr photoperiod, a constant temperature of 25 ± 1 °C and a relative humidity of 95-100 %. After 2 weeks in the dew chamber 4 plantlets from each of the in vitro treatments were harvested, 8 plantlets from each treatment were placed into a growth chamber and 8 plantlets were left in the dew chamber. The growth chamber had a light intensity of 90 µmol s^{-1} m^{-2} cool-white fluorescent light, a 16 hr photoperiod, a constant temperature of 25 °C and a relative humidity which ranged between 30 - 55 %. After 17 and 21 days ex vitro 4 plantlets were harvested from each treatment, both from the dew and growth chambers. The leaves on these transplants were identified as P leaf (persistent leaf from culture), leaf 1 (first new leaf formed after transplantation), leaf 2 (second new leaf), etc. Two leaves of each type were randomly selected from the four plantlets from each treatment. Two epidermal peels were removed from each of the leaves sampled for evaluation of stomatal function.

Stomatal function was determined using the 1M NaCl solution as described for Experiment 2. Once stomatal closure occurred or if the protoplasm collapsed an attempt was made to reopen the stomata by replacing the 1M NaCl solution

with the phosphate buffer. Photomicrographs were taken while the epidermal peels were in buffer, after the application of the hypertonic (1M NaCl) solution and again once the hypertonic solution was replaced by the buffer. To ensure precision in measurement of stomatal aperture a 400-480 magnification was used. Each photomicrograph contained no more than 5-8 stomata. The number of stomata measured for each leaf type of every treatment ranged from 5-20 and was limited because of time considerations and the problem of guard cells going out of focus while in the hypertonic solution. The stomatal apertures were measured as in the previous experiment. An aperture of $\leq$ 1 µm was considered closed. This was a conservative value since a stomatal aperture $\leq$ 2 µm was usually considered to be closed (Zelitch, 1961; cited in Brainerd and Fuchigami, 1981).

4.2.7. The effects of high relative humidity and low light intensity on stomatal function of greenhouse-grown 'Silvan' plants.

Six week old greenhouse-grown source plants were placed in the same dew chamber used for the transplants from culture. At weekly intervals the newly-formed leaves were tagged. After 4 weeks the original leaves present on the plant when it was transferred to the dew chamber and the new leaves formed during the second week in the dew chamber were harvested. Two epidermal peels were removed from each of these leaves and stomatal function was determined using the 1M NaCl test. Statistical analysis of all data was done using PC-SAS. Differences between means were tested using the Student's t-test.

4.3. RESULTS AND DISCUSSION

4.3.1. The effect of medium agar concentration and in vitro rooting on 'Silvan' following transplantation.

In experiment 1 transplant survival was 98 % during the first 2 weeks. All of the shoots and plantlets in experiment 2 survived in the 2 week test and all plantlets survived in the 3 and 4 week tests. However, the survival of shoots was not as good in the 3 (1/30) and 4 (5/15) week tests (Table 3). 'Silvan' shoots were apparently less able than plantlets to adapt to the ex vitro environment. The cause for the lack of shoot survival once the covers were removed is not fully understood but it is postulated that the new roots formed ex vitro were unable to compensate for the sudden increase in the transpiration rate once these covers were removed.

In contrast to results with carnation (Ziv et al, 1983) and cherry rootstock (Marin and Gella, 1987) where increasing the medium agar concentration from 6 g/l to 14-15 g/l increased ex vitro survival, 'Silvan' blackberry survival ex vitro was not affected by the level of agar in the medium. The survival of rooted treatments was least affected by the early removal of the flat covers in experiment 3 (Table 3). This suggests that despite possible evidence for non-functional in vitro-formed roots (Grout and Aston, 1977, Logan and Zettler, 1985, Skolmen and Mapes, 1978, Debergh and Maene, 1981), roots of 'Silvan' formed in vitro are probably functional after transplantation. Loganberry plantlets (Rubus loganobaccus) were also hardier after 1 month ex vitro than shoots (Donnelly et al, 1980).

At the time of transplantation mean transplant height was similar for all treatments (1.1 cm) except treatment 5 (0.9 cm) (Figure 2). The apparent decrease in height from transplantation (week 0) to week 1 resulted from an

undesirable but necessary change in measurement practice implemented to reduce handling of the transplants. The plantlets from media containing 6 and 9 g/l agar concentrations were significantly taller than the shoots throughout the 3 week period. This was true for the first but not the second and third week for the highest medium agar concentration (12 g/l). For the first 2 weeks after transplantation, plantlets from media containing 6 g/l were tallest (2.04 cm) but the mean height of all rooted transplants were statistically similar by the end of tne third week, ranging from 2.84, 2.89 and 2.27 cm for medium agar concentrations of 6, 9 and 12 g/l respectively.

Plantlets and shoots all had 7-8 leaves at transplantation (Figure 3). The initial reduction in leaf number from week 0 to week 1 was due to the death of persistent leaves. At the end of the first week plantlets had significantly more leaves than shoots from the same agar concentrations. All treatments had a similar mean leaf number at the end of the second (7) and third week (8-9) except for treatment 5 which had 6 (second week) and 7 (third week). By the end of the third week almost all persistent leaves had abscissed.

One week after transplantation persistent leaves from culture were still the longest leaves on the transplants and had not grown. The average persistent longest leaf lengths were found on plantlets from the 6 and 9 g/l agar concentrations (1.40 and 1.35 cm) (Figure 4). Shoots from 6 and 9 g/l as well as plantlets from 12 g/l agar concentrations had similar (ranging from 1.05 - 1.75 cm) average persistent longest leaf lengths at this time, while shoots from 12 g/l agar concentrations (0.98 cm) had the shortest persistent leaves. At the end of the second and third week plantlets did not differ in average longest new leaf lengths regardless of the agar concentration in the medium from which they were transferred. However, plants that were rooted at the time of

Table 3. Mean initial (week 0) and final (week 3) fresh weights (g) of 'Silvan' transplants from medium agar concentrations of 6, 9 and 12 g/l.

Treatment	week 3		
	Fresh Weight (g)		
	Initial	Final	N*
6 g/l agar. shoots	0.16 b	0.19 b	1/10
6 g/l agar, plantlets	0.24 a	0.96 a	10/10
9 g/l agar, shoots	0.24 a		0/10
9 g/l agar, plantlets	0.18 ab	0.83 a	10/10
12 g/l agar, shoots	0.15 b		0/10
12 g/l agar, plantlets	0.13 b	0.39 b	10/10

Duncan's groupings at the 0.5 % level of significance.
* N = survival; the denominator indicates the initial sample size.

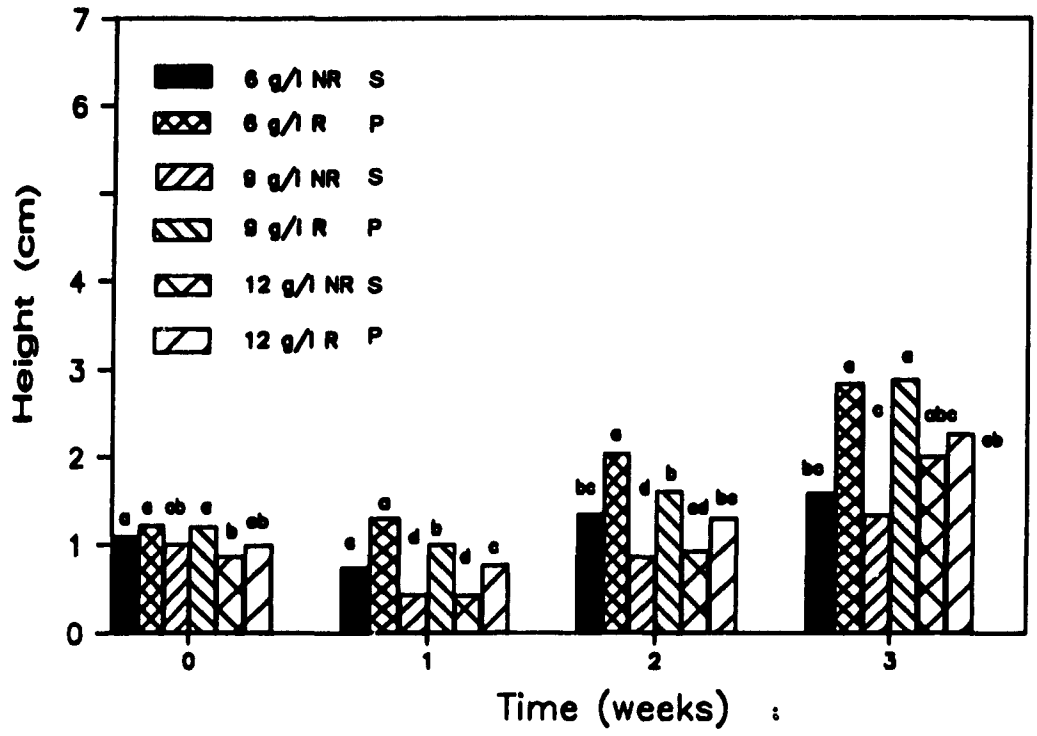


Figure 2. Mean height of 'Silvan' blackberry shoots (S) and plantlets (P) rown on medium containing 6, 9 and 12 g/l Difco-bacto agar, during the first 3 weeks after transplantation. Comparison of means was done within weeks.

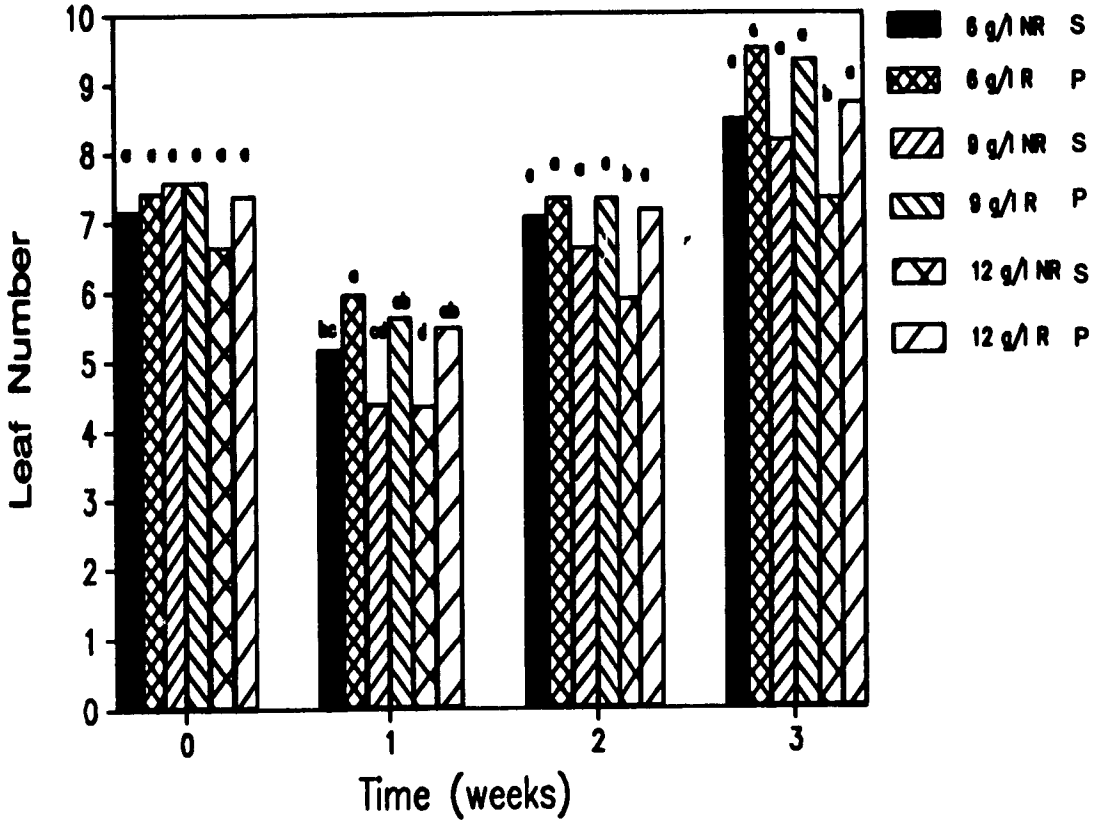


Figure 3. Mean leaf number of 'Silvan' blackberry shoots (S) and plantlets (P), grown on media containing 6, 9 and 12 g/l Difco-bacto agar, during the first 3 weeks after transplantation. Comparison of means was done within weeks.

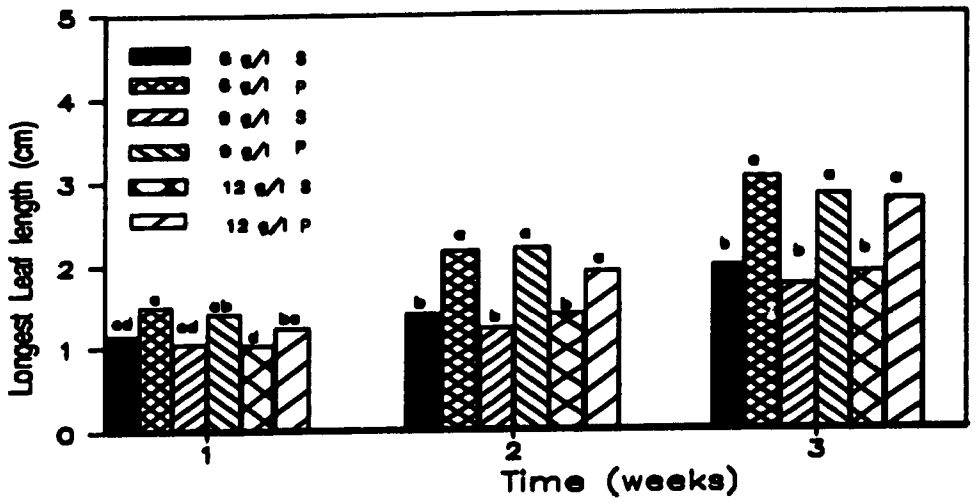


Figure 4. Mean longest leaf length of 'Silvan' blackberry shoots (S) and plantlets (P), grown on media containing 6, 9 and 12 g/l Difco-bacto agar, during the first 3 weeks after transplantation. Comparison of means was done within weeks.

transplantation (plantlets) had significantly longer new leaves (2 - 3 cm) than non-rooted shoots (1.2 - 2 cm).

Ex vitro shoot height, leaf number and root length of cauliflower and chrysanthemum decreased with increasing medium agar concentrations. However, the concentrations of agar used were higher than those used in this experiment (12, 16 and 20 g/l) (Short et al., 1987). The negative effect of high agar concentrations on shoot height, leaf number and root length was attributed to the decrease of water availability in the culture vessels. Both reduced water (Debergh, 1983) and mineral (Tanaka, 1981) availability in media occurred through the decreased pore size and increased matric potential at high agar concentrations. The point at which the agar concentration negatively effects plant growth varies with different brands of agar (Debergh, 1983). It is possible that increased in vitro root absorption might counteract the reduced water and nutrient availability in such cases. This could perhaps explain why in vitro-rooted plantlets, at the highest agar concentration (12 g/l), developed more new leaves and leaves of longer length after 3 weeks ex vitro than shoots grown on the same agar concentration (Figures 2-4).

In vitro rooting had a much greater effect than did medium agar concentration on performance of ex vitro transplants. 'Silvan' blackberry plantlets were taller and had greater longest leaf lengths than shoots during the first 3 weeks ex vitro (Figure 2 and 4) suggesting that ex vitro plantlets were more immediately productive than shoots. In vitro-formed roots of 'Silvan' blackberry were apparently functional under ex vitro conditions.

4.3.2. The effect of fresh weight at transplantation (initial) and persistent leaf number on final fresh weight 3 weeks after transplantation.

Three weeks after transplantation the mean initial fresh weights of treatment 2 (0.24 g) and treatment 3 (0.24 g) were significantly greater than treatments 1 (0.16 g), 5 (0.15 g) and 6 (0.13 g) but similar to treatment 4 (0.18 g) (Table 3). At the end of the 3 week period the average fresh weights of treatments 2 (0.96 g) and 4 (0.83 g) were significantly greater than treatments 1 (0.19 g) and 6 (0.39 g). The net growth increase was statistically similar between all surviving treatments: 1 (195 %), 2 (466 %), 4 (499 %) and 6 (308 %) (Table 4). Almost no shoots survived the change in environment once the covers were removed. This seems to indicate that the shoots were not as acclimatized as the plantlets 2 weeks following transplantation.

At the end of 3 weeks ex vitro, treatment 5 had the fewest persistent leaves (5.6). Treatments 1 (5.7), 5 (5.6) and 6 (5.9) had fewer persistent leaves than treatments 2 (7.6) and 4 (7.6). No relationship was observed between above-ground fresh weight of plants harvested 3 weeks after transplantation and initial persistent leaf number.

Romberger and Tabor (1971) discussed the possibility that high agar concentrations in media may reduce the diffusion rates of enzymes and other large molecules. This, as well as the possibility of lower water potential (Short et al., 1987)(Ziv et al., 1983), may have contributed to the negative effect of high agar concentrations on initial and final fresh weights of cauliflower and carnation. Increased agar (Difco-Bacto) concentrations (6 - 8 g/l) significantly reduced the fresh weight of Cynara scolymus L.. However, there were no significant differences in the fresh weight production of C. scolymus L. shoots grown on 8 g/l to 15 g/l agar concentrations (Debergh, 1983).

Table 4. Mean persistent leaf number at transplantation and the increase in growth (%) of 'Silvan' blackberry transplants grown on medium agar concentrations of 6, 9 and 12 g/l from transplantation to week 3.

Treatment	week 3	
	P leaf	Growth (%)
6 g/l agar, shoots	5.8 b	195 a
6 g/l agar, plantlets	7.6 a	466 a
9 g/l agar, shoots	6.6 ab	
9 g/l agar, plantlets	7.6 a	499 a
12 g/l agar, shoots	5.6 b	
12 g/l agar, plantlets	6.0 b	308 a

Duncan's groupings at the 0.5 % level of significance

No relationship was found between the mean initial and final fresh weights of plantlets from treatments 2 and 6 of the 3 week test. However, a linear relationship (r^2 = 0.52) was found between the mean initial and final fresh weights of plantlets from treatment 4. Despite results from ex vitro rooting and acclimatization experiments with Pelargonium zonale plantlets demonstrating that plant size (greater than 2 cm in height) and leaf size were very important factors to consider when transplanting (Aldrufeu, 1987), no relationship was found between the mean initial leaf number and final fresh weight of 'Silvan' plantlets in any of the treatments. This was surprising since it would be logical to assume that a greater initial fresh weight at transplantation would provide more resources in the form of stored materials or photosynthetic area for new leaf growth. Since no relationship was found between initial leaf number and final fresh weight there is no substantiation from this work that persistent leaves of 'Silvan' blackberry mobilize stored elements for areas of new growth as suggested for Chrysanthemum morifolium (Wardle et al., 1983).

In contrast to results with 'Silvan' blackberry, Short et al. (1987) found that increasing the agar concentration decreased leaf number in cauliflower and chrysanthemum plantlets. However, as mentioned previously, the starting point for their analysis was 12 g/l increasing to 20 g/l. High agar concentrations (10-20 g/l) were shown to reduce the number and length of in vitro roots in jackfruit (Artocarpus heterophyllus Lam.) (Rahman and Blake, 1988) and shoot height and root length in chrysanthemum (Short et al., 1987).

4.3.3. The effect of medium agar concentration and in vitro rooting on stomatal index

The mean stomatal index (S.I.) of persistent leaves of transplants (from experiment 1) ranged from 18.2 (treatment 5) to 22.8 (treatment 2) (Figure 5). The S.I. of persistent leaves from treatments 2 (22.8), 3 (20.6) and 6 (20.5) were similar as were treatments 1 (19.2), 3 (20.6), 4 (19.2) and 5 (18.2). The mean S.I. of leaves formed during the first week (week 1) of treatments 1 (20.4), 2 (22.5), 3 (21.6), 4 (21.26) and 5 (20.5) were not significantly different. The S.I. of treatment 6 (23.2) was significantly greater than treatments 5 and 1. New leaves developed during the second and third week on all treatments had similar S.I., ranging from 20.6 to 23.1 for new leaves formed the second week and 21.2 to 22.9 for new leaves formed the third week. The S.I. of persistent compared with new leaves increased significantly in treatments 1: 19.2 to 22.7 (week 2); 4: 19.2 to 21.8 (week 3); and 5: 18.2 to 22.5 (Figure 6). Treatments 2, 3 and 6 showed no significant change in S.I. between persistent and new leaves formed up to 3 weeks after transplantation. The S.I. of leaves from greenhouse-grown 'Silvan' was 14.8.

There was no relationship found between medium agar concentration or in vitro rooting and S.I.. A higher S.I. when the stomata were non-functional, could have adversely affected the ability of transplants to acclimatize. However, in 'Silvan' blackberry in vitro-rooting apparently played a much greater role than S.I. in determining the ability of plants to acclimatize ex vitro since no particular trend was found between treatments and stomatal index.

Unlike the S.I. of leaves of micropropagated Leucaena leucocephala (Lam) De Wit. which was lower (7.21) than on leaves from the original, mature donor plant (12.02) (Dhawan and Bhojwani, 1987) or potato (Conner and Conner, 1984) and rose plants (Queralt, 1988) whose S.I. was unaffected by micropropagation, the S.I. of leaves from mature greenhouse-grown 'Silvan' plants was lower (14.8) than that of persistent leaves and all leaf types following transplantation.

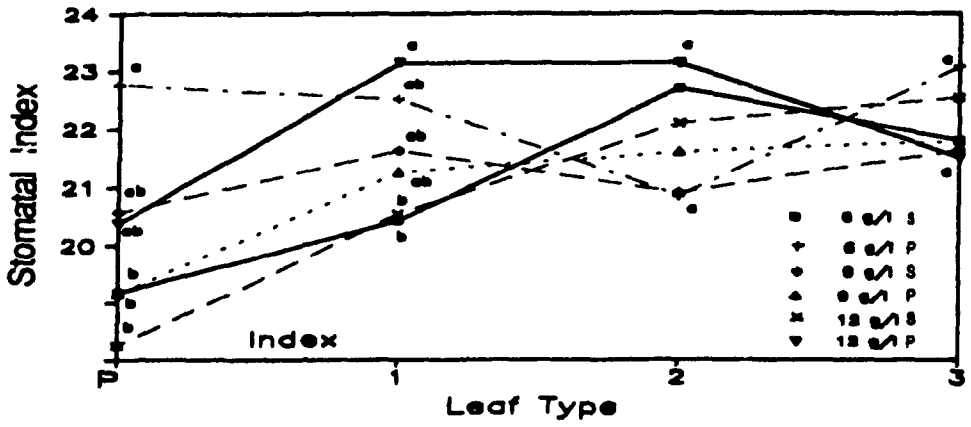


Figure 5. Mean stomatal index of leaves from 'Silvan' blackberry shoots (S) and plantlets (P) grown on media containing 6, 9 and 12 g/l Difco-bacto agar. These leaves had developed in culture (P) and during the first (wk 1), second (wk 2) and third (wk 3) week after transplantation.

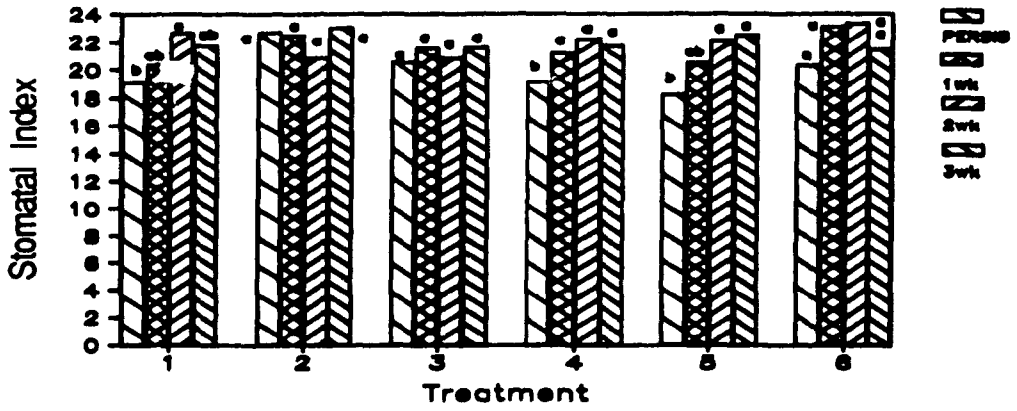


Figure 6. Comparison within agar and in vitro-rooting treatments of mean stomatal index of 'Silvan' blackberry shoots and plantlets from media containing 6, 9 and 12 g/l Difco-bacto agar. Sampling included leaves which had developed in culture (Persis) and during the first (1 wk), second (2 wk) and third (3 wk) week after transplantation.

4.3.4. Evaluation of ex vitro stomatal function of 'Silvan' plants

The majority of stomata on epidermal peels of greenhouse-grown 'Silvan' leaves were open after the peels had floated for 30 min on the phosphate buffer under the sodium light. Stomata closed after exposure to the ABA solution for 25 min under the sodium light. They did not reopen after 20 min of floating on the phosphate buffer under light. The stomata closed after 35 min when the epidermal peels were floated on the phosphate buffer and the petri dishes darkened with aluminium foil. Subsequent stomatal reopening occurred within 20 min when the epidermal peels were put back under the sodium light. The stomata closed within 20 sec when epidermal peels were floated on the 1M NaCl solution, and reopened within 1 min once the 1M NaCl solution was replaced with the phosphate buffer. Due to its rapidity, the 1M NaCl treatment was chosen as the most time-efficient test for the purpose of determining stomatal function (open-closure mechanism).

Inconsistencies in stomatal aperture (S.A.) measurements occurred when the phosphate buffer was reapplied after the 1M NaCl treatment. In some cases the final S.A. were less than the original ones. Perhaps all the NaCl solution was not completely removed or the epidermal peels had moved so S.A. measurements were not of the same stomatal populations. The adding and diluting of the NaCl solution caused stomata to open and close several times. However, reopening was impaired after a second 1M NaCl application. This was thought to be due to the incomplete removal of all salt when replacing the NaCl solution with the phosphate buffer.

4.3.5. Evaluation of stomatal closure of the first new leaves of 'Silvan' plantlets three weeks following transplantation

No stomatal closure occurred on persistent or new leaves in any treatment at the end of the 2 week test. At the end of the 4 week test all leaves which were not senescing had stomata which could close in response to the 1M NaCl solution and reopen when floated on buffer (all of the persistent and many week 1 leaves exhibited senescence).

Original medium agar concentration had no apparent effect on stomatal function so all treatments from the 3 week test were pooled. Three weeks after transplantation persistent leaves had an initial S.A. of 5.2 ± 0.7 µm and this was basically unchanged after application of the 1M NaCl solution where the mean S.A. was 4.8 ± 1.0 µm (Table 5). The leaves which developed during the first week (week 1) had an initial S.A. of 4.7 ± 1.2 µm. This S.A. decreased to 2.7 ± 0.6 µm after the 1 M NaCl solution was applied. The new week 2 leaves had an initial S.A. of 4.0 ± 1.0 µm which completely closed to 1.5 ± 0.5 µm after the NaCl solution was applied. The new week 3 leaves had an initial stomatal aperture of 4.9 ± 0.8 µm. After the NaCl solution was applied most stomata completely closed to 0.4 ± 0.3 µm. Stomata from all new leaves were able to subsequently reopen when the epidermal peels were floated on the phosphate buffer. The potential for stomata to achieve complete closure was transitional during the first 3 weeks of ex vitro acclimatization (Table 6). Each successive new leaf formed under the new environmental conditions had improved stomatal closure.

A few stomata of the last persistent leaves retained after 3 weeks ex vitro exhibited limited stomatal response to the NaCl solution in that they were able to close significantly. This occurred in about 40 % of the epidermal peels evaluated. It is possible that only persistent leaves which were not fully

developed at transplantation and whose development overlapped both in vitro and ex vitro environments were able to develop partial stomatal function. The persistent leaves which developed last and some new week 1 leaves may be included in this group.

After several days of exposure to relative humidity levels of 30-45 %, stomata on persistent leaves of apple closed more completely (85 %) 15 min after excision than persistent leaves exposed to low humidity levels for 4 days (15 %) or less (5 %) (Brainerd and Fuchigami, 1981). The percent or speed (rate) of stomatal closure of persistent cherry leaves (98 %) was considerably greater than in vitro cherry leaves (72 %) when exposed to 45 % relative humidity levels for 15 min. (Marin et al. 1988). Stomatal conductance of apple and cherry was significantly less (up to 70 %) on leaves gradually exposed to relative humidity levels of 65 % over a 12 day period prior to sampling. It has been observed in other plant species that the new leaves formed during the acclimatization period exhibited improved capacity for stomatal closure when compared to leaves from culture (Conner and Conner, 1984, Dhawan and Bhojwani, 1987, Marin et al, 1988, Marin and Gella, 1988). However, this was usually determined by measuring the amount of water lost over time after leaf detachment not by actual stomatal aperture measurements. Stomatal closure could have been due to membrane collapse, especially when leaves were exposed to very low relative humidity levels. The above authors did not verify whether these stomata could subsequently reopen.

Table 5. Mean initial stomatal aperture (± standard error) in buffer and the stomatal aperture after replacing the buffer with a 1 M NaCl solution on leaf peelsof persistent and new leaves of 3 week old 'Silvan' blackberry plantlets. New leaves developed during the first, second and third week after transplantation.

Leaf	Buffer (um)	1 M NaCl (um)
0 week	5.2 ± 0.7	4.8 ± 1.0
1st week*	4.7 ± 1.2	2.7 ± 0.6
2nd week	4.0 ± 1.0	1.5 ± 0.5
3rd week	4.9 ± 0.8	0.4 ± 0.3

* Time period during which the new leaf developed.

4.3.6. The effects of high relative humidity and low light intensity on ex vitro stomatal function of 'Silvan' plantlets grown on full or 1/4 strength rooting media

After 14 days in the dew chamber persistent leaves of the "Full MS" treatment plants had an initial S.A. of 4.47 µm and the S.A. of the new leaves varied from 4.61 µm (leaf 1) to 3.38 µm (leaf 2) to 3.75 µm (leaf 3) (Table 6). Once the NaCl solution was applied, the first new leaf and the third new leaf showed significant reductions in mean S.A. (3.27 µm and 1.75 µm, respectively). Only in the third new leaves was stomatal closure complete. Stomata of persistent and new leaves reopened once the NaCl solution was replaced with buffer solution. Reopened stomatal apertures of new leaves ranged from 3.36 µm (leaf 2) to 3.64 µm (leaf 1) and 4.61 µm for persistent leaves.

After 14 days in the dew chamber persistent leaves of the "1/4 MS" treatment plants had an initial S.A. of 3.8 µm and the initial S.A. of the new leaves ranged from 3.89 µm (leaf 2) to 4.11 µm (leaf 3) to 4.92 µm (leaf 1). As in the "Full MS" treatment, when the NaCl solution was applied only the first new leaves showed a significant reduction in S.A. (3.53 µm). Stomatal closure was incomplete in persistent leaves (3.00 µm) and all new leaves: 3.29 µm (leaf 2) and 3.48 µm (leaf 3). Reopened stomatal apertures of new leaves ranged from 3.92 µm (leaf 2) to 4.59 µm (leaf 3) and 3.81 µm for persistent leaves.

After 14 days under conditions of high humidity stomata of leaves of plants grown on the "Full MS" treatment appeared to be more functional than stomata of leaves of plants grown on the "1/4 MS" medium. However, only stomata from the third new leaf of plants grown on "Full MS" treatment plants was completely closed (1.75 µm). Stomata of leaves from shoots or plantlets grown

on "1/4 MS" treatment could not generally close beyond 3 µm.

After 17 days in the dew chamber the persistent leaves from plants grown in the "Full MS" treatment had an initial S.A. of 3.63 µm and the S.A. of new leaves ranged from 3.80 µm (leaf 2) to 4.46 µm (leaf 3) to 6.08 µm (leaf 1). After application of the NaCl solution all new leaves had significantly reduced S.A. which ranged from 2.56 µm (leaf 2) to 2.27 µm (leaf 3) to 4.29 µm (leaf 1) although stomatal closure was incomplete in all cases. Once the NaCl solution was replaced with the buffer, the stomata of persistent leaves (3.93 µm) and the first (5.73 µm), second (3.63 µm) and third (3.95 µm) new leaves could all reopen.

After 17 days in the dew chamber the persistent leaves of the "1/4 MS" treatment had a mean S.A. of 3.18 µm. The first new leaf had an initial S.A. of 4.75 µm and the second, 4.54 µm. After application of the NaCl solution stomata from both the first (3.14 µm) and second (2.27 µm) new leaves had significantly reduced apertures. However, complete closure was not observed in any case. After replacement of the NaCl solution with buffer the stomata of the first (4.58 µm) and second (4.69 µm) new leaves reopened.

After 14 days in the dew chamber and 3 days in the growth chamber stomata of new leaves grown on the "Full MS" treatment had initial S.A. which varied from 4.15 µm (leaf 1) to 2.82 µm (leaf 2) to 2.35 µm (leaf 3). After application of the NaCl solution, the mean S.A. were significantly reduced on leaf 1 (3.35 µm) and leaf 3 (1.45 µm) but not on leaf 2 (2.09 µm). Stomata from both the second and third new leaves were completely closed. Once the NaCl solution was replaced by buffer the stomata of the first (4.17 µm), second (3.42 µm) and third (3.05 µm) new leaves reopened.

After 17 days, the initial S.A. of stomata on leaves of plants grown on the "Full MS" treatment in the growth chamber were smaller, ranging from 2.35

µm to 4.15 µm, compared with plants from the same "Full MS" treatment in the dew chamber, where the S.A. ranged from 3.63 µm to 6.08 µm. The second and third new leaves from plants grown on "Full MS" medium plants in the growth chamber had smaller S.A. after the NaCl solution was applied than their counterparts in the dew chamber. The stomata of the second (2.95 µm) and third (1.75 µm) new leaves of plants grown on the "Full MS" medium and in the dew chamber for 17 days were able to close to a smaller aperture than the stomata on the second (3.29 µm) and third (3.48 µm) new leaves of plants grown on the "1/4 MS" medium. Unfortunately, the data for day 17 of "1/4 MS" plants in the growth chamber cannot be presented.

After 21 days in the dew chamber the persistent leaves of the "Full MS" treatment plants had an initial S.A. of 3.78 µm. Stomata on the new leaves had initial S.A. ranging from 3.27 µm (leaf 3) to 4.31 µm (leaf 2). After the NaCl solution was applied the S.A. of the persistent leaves was significantly reduced (2.2 µm) (close to full closure). The S.A. of the new leaves after the application of the NaCl solution was also significantly reduced ranging from 1.45 µm (leaf 3) to 3.96 (leaf 2). However, only the third new leaf had stomata which were fully closed (1.45 µm). After replacing the NaCl solution with buffer, stomata on the persistent leaves reopened (3.93 µm) as did stomata on the third (4.24 µm) and fourth (2.98 µm) new leaves. Surprisingly, stomata on the first (3.11 µm) and second (3.36 µm) new leaves closed further.

After 21 days in the dew chamber the initial S.A. of the persistent leaves of plants grown on the "1/4 MS" medium was 5.45 µm. The initial S.A. of stomata of new leaves ranged from 3.18 µm (leaf 3) to 4.36 µm (leaf 1). After the NaCl solution application, the stomata of the persistent leaves had a significantly reduced S.A. of 4.54 µm. Stomata from all the new leaves had significantly reduced apertures after the NaCl solution was applied, ranging from

1.74 µm (leaf 4) to 2.90 µm (leaf 1). Only the second and fourth leaves had stomata which could completely close (1.74 µm and 1.81 µm). Once the NaCl solution was replaced with buffer, stomata of persistent and new leaves reopened to apertures which ranged from 2.95 µm (leaf 3) to 5.00 µm (P leaf)

Twenty-one days after transplantation, the stomata on the persistent leaves of plants grown on the "Full MS" medium, which had been in the growth chamber for 1 week, were initially closed (0.98 µm) but they closed even further (0.15 µm) in response to the NaCl solution. The initial S.A. of stomata of new leaves ranged from 2.82 µm (leaf 5) to 3.48 µm (leaf 4). Stomata of most leaves had significantly reduced apertures and most closed after the NaCl solution was applied. After the NaCl solution was replaced with buffer, stomata on persistent (2.72 µm), second (3.03 µm), third (2.30 µm), fourth (3.33 µm) and fifth (2.52 µm) new leaves could reopen but stomata on the first new leaf (1.71 µm) could not.

The stomata of persistent leaves from the "1/4 MS" treatment plants, which had been in the growth chamber for 1 week, had an initial S.A. of 1.29 µm. The initial S.A. of stomata of new leaves ranged from 2.23 µm (leaf 2) to 2.27 µm (leaf 3). After the application of the NaCl solution, stomata of the persistent leaves had a significantly reduced S.A. of 0.36 µm (closed). The stomata on the first new leaf did not fully close (2.27 µm) but stomata on the second and third new leaves also had significantly reduced apertures and they closed to 0.82 and 1.01 µm, respectively. Once the NaCl solution was replaced, stomata on persistent leaves could reopen to some extent but the S.A. was still considered to be closed (1.21 µm). Stomata of the first (4.54 µm), second (2.5 µm) and third (2 µm) new leaves reopened in response to the replacement of the NaCl solution but the S.A. of stomata of the third leaf remained fully closed, and could not reopen.

Stomata of both persistent and new leaves had larger mean initial apertures when plantlets were grown in the dew chamber for 21 days than when they were transferred to the growth chamber for 1 week after having grown in the dew chamber for 2 weeks. Though all stomata from leaves of plants in the dew chamber had significantly reduced apertures in response to the NaCl solution, the resulting aperture was always greater than that of stomata on leaves from plants in the growth chamber. These trends were the same for plants from both "Full MS" and the "1/4 MS" treatments. Based on these observations, it appears that the environment, particularly relative humidity in conjunction with low light intensity, plays an important role in determining the capacity for stomata to function.

The stomata of persistent leaves from the "Full MS" treatment plants in the growth chamber had a much decreased initial S.A. (0.98 μm) compared with their counterparts in the dew chamber (3.78 μm). Similarly, after the NaCl solution had been applied, the S.A. of the persistent leaves from the growth chamber (0.15 μm) was smaller than that of the persistent leaves from the dew chamber (2.2 μm). The persistent leaves from the "1/4 MS" treatment in the growth chamber also had reduced initial S.A. (1.29 μm) (closed) when compared with the persistent leaves from the same medium treatment in the dew chamber (5.45 μm). This trend held true both after the NaCl was applied and on its removal.

The first new leaf had initial S.A. greater than 4 μm for all harvest dates and treatments except for the treatment placed in the growth chamber for one week. Only the stomata of leaves from 21 day plants which had been in the growth chamber for 1 week could close after the NaCl solution was applied (1.70 μm). However, after removal of the NaCl solution the S.A. did not increase (1.71 μm). So, the stomata in this case were unable to reopen. There

did not seem to be any trend regarding the failure of stomata to reopen. The stomata on the first new leaves of the "1/4 MS" treatment plants had reduced initial apertures when placed in the growth chamber for a week. The majority of stomata from the first new leaves of all treatments had significantly reduced S.A. even if they could not fully close.

Stomata from the second new leaves of the "Full MS" treatment plants had initial apertures ranging from 2.82 μm, for the leaves of plants which had been placed in the growth chamber for 3 days, to 4.31 μm for leaves of plants which had been left in the dew chamber for 21 days. When comparing the ability for stomata from the first and second new leaves of the "Full MS" treatment plants to close in response to the NaCl solution, stomata of the second new leaves appeared to respond to the change (decrease) in relative humidity sooner than stomata of the first new leaves. After 3 days in the growth chamber stomata of the second new leaves had smaller S.A. (2.09 μm) than their counterparts in the dew chamber (2.56 μm) in response to the NaCl solution. However, no appreciable difference in S.A. was observed in stomata of the first new leaves under the same conditions. Like the first new leaves on plants grown on the "Full MS" medium, stomata of the second new leaves of plants grown on the "1/4 MS" medium had reduced initial S.A. when plants were transferred to the growth chamber for 1 week. This trend continued after the NaCl solution was applied and also after it was replaced by the buffer. Only stomata from the second new leaves of the "Full MS" treatment plants transferred to the growth chamber and 14 day plants from the "Full MS" and "1/4 MS" treatment plants failed to close significantly after application of the NaCl solution.

The third new leaves of the "Full MS" treatment plants in the dew chamber had initial S.A. which were smaller on day 21 (3.27 μm) than day 17 (4.46 μm). This was also true after the NaCl solution was applied. The same

trend was observed for the third new leaves from plants of the same treatments but transferred to the growth chamber. Stomata on third new leaves of plants placed in the growth chamber for 3 days and on leaves of plants from the "Full MS" treatments in the dew chamber for 21 days were able to completely close (apertures less than 2 μm). The third new leaves on plants grown on the "1/4 MS" treatment had improved stomatal function when placed in a growth chamber for a week compared with the third new leaves of plants left in the dew chamber. The third new leaves of plants from the "1/4 MS" treatment which were left in the dew chamber were not expanded enough at day 17 to sample. The plants grown under the "1/4 MS" treatment were slower in producing new leaves ex vitro.

Stomata on each successive new leaf had a greater capacity to open and close both on plants growing in the growth and dew chambers (Table 6). So, stomatal function was transitional (Figure 7) whether the relative humidity level was high or not. However, under conditions of low relative humidity (ie transferred to the growth chamber), stomatal apertures were reduced in all leaves with complete closure occurring in response to 1M NaCl in all leaf types (persistent to leaf 4-5) regardless of the media treatment (ie "Full MS" or "1/4 MS") on which the plantlets were grown. This was in contrast to stomata on the persistent, first and second new leaves, developed under high humidity conditions, which after 21 days could not close. Therefore, it appears that ex vitro environmental factors such as relative humidity and light levels are more important than in vitro media composition in determining the functional capacity of stomata after transplantation.

Diluting the basal medium for the "1/4 MS" treatment did not improve stomatal function of ex vitro 'Silvan' blackberry leaves when compared with leaves of plantlets grown on the "Full MS" treatment. It is appears contrary

to protocols developed for in vitro acclimatization of red raspberry which recommend placing plantlets in 1/5 strength medium 2 weeks prior to transplantation (Welander, 1985). It is possible that the 3 weeks on 1/4 strength medium used in this experiment resulted in nutrient depletion. However, the blackberry plantlets appeared vigorous and healthy at transplantation. Reducing the sugar content may promote photoautotrophy in certain plants (Grout and Price, 1987, Kozai, 1988a) but it did not appear to improve stomatal function in `Silvan' blackberry.

The ability of stomata to function is related to their structural or anatomical features. Guard cell walls of cherry were thinner in vitro than on greenhouse-grown or field-grown plants (Marin et al., 1988). In vitro stomata of cherry also lacked invaginations in the epidermal cells next to the inter-guard cell wall ends which were evident in acclimatized leaves. The arrangement of microfibrils of in vitro guard cell walls did not appear altered when compared to that of stomata of greenhouse-grown leaves. (Marin et al., 1988). Anatomical changes which might have occurred in the anatomy of `Silvan' blackberry stomata were beyond the scope of this experiment. However, when examining epidermal peels, it was observed that even if stomata did not close during the application of the 1 M NaCl solution the guard cell protoplasm collapsed suggesting that the guard cell membrane was permeable and that the guard cell was physiologically functional. The protoplasm of in vitro stomata of Chrysanthemum morifolium (Wardle and Short, 1983) and carnation Dianthus caryophyllus L. (Ziv et al., 1987) collapsed in a similar way in response to ABA and 0.8 M sucrose. Wardle and Short (1983) suggested that, during stomatal development, the deposition of cellulose and microfibrils in the cell walls may dictate the "closed" position of the guard cells. The closed state of in vitro stomata is partially

Table 6. Mean stomatal apertures of 'Silvan' blackberry plantlet leaf peels in the buffer (B), after a 1 M NaCl solution was drawn over the peel (S) and once the salt solution had been replaced with buffer(B2). Stomata were considered closed if the aperture was ≤ 2 µm.

		P leaf	leaf 1	leaf 2	leaf 3	leaf 4	leaf 5
				Full MS (Dew Chamber)			
Day 14	B	4.47	4.61	3.38	3.75		
	S	4.08	3.27**	2.95	1.75**		
	B2	4.61	3.64	3.36	3.52		
Day 17	B	3.63	6.08	3.80	4.46		
	S	3.11	4.29**	2.56**	2.27**		
	B2	3.93	5.73	3.63	3.95		
Day 21	B	3.78	4.27	4.31	3.27	3.40	
	S	2.20**	3.42**	3.96**	1.45**	2.82**	
	B2	3.93	3.11	3.36	4.24	2.98	
				Full MS (Growth Chamber)			
Day 17	B		4.15	2.82	2.35		
	S		3.35*	2.09	1.45**		
	B2		4.17	3.42	3.05		
Day 21	B	0.98	2.90	3.03	2.16	3.48	2.82
	S	0.15**	1.70**	1.21	0.70**	0.25**	0.40**
	B2	2.72	1.71	3.03	2.30	3.33	2.52
				1/4 MS (Dew Chamber)			
Day 14	B	3.80	4.92	3.89	4.11		
	S	3.00	3.53**	3.29	3.48		
	B2	3.81	4.27	3.92	4.59		
Day 17	B	3.18	4.75	4.54			
	S	3.18	3.14**	2.72**			
	B2	4.58	4.69				
Day 21	B	5.45	4.36	3.88	3.18	4.24	
	S	4.54*	2.90**	1.74**	2.27**	1.81**	
	B2	5.00	4.72	3.40	2.95	3.03	
				1/4 MS (Growth Chamber)			
Day 21	B	1.29	2.98	2.23	2.27		
	S	0.36**	2.27**	0.82**	1.01**		
	B2	1.21	4.54	2.50	2.00		
				Greenhouse-grown (Dew Chamber)			
		Old Leaf	Week 2				
Day 21	B	2.70	5.08				
	S	1.51**	5.31				
	B2	1.60	3.92				

** Significant at the 0.05 level using student's t-test
* Significant at the 0.1 level using student's t-test

open. Further stomatal opening of in vitro stomata of chrysanthemum and subsequent reclosure to the original aperture could be induced but further closure could not (Wardle and Short, 1988).

4.3.7. The effects of high relative humidity and low light intensity on stomatal function of greenhouse-grown 'Silvan' plants

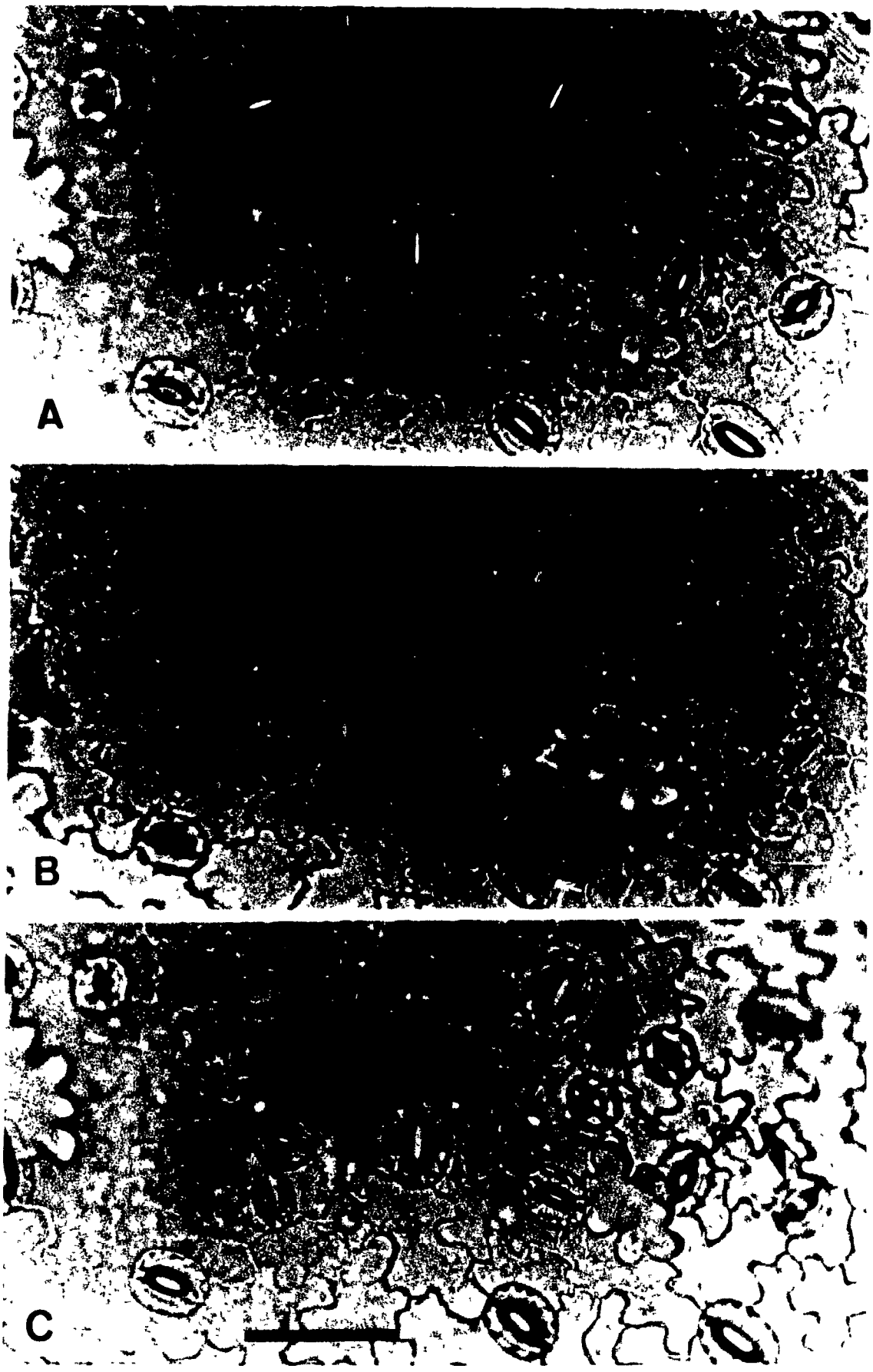
Examined 4 weeks after the greenhouse-grown 'Silvan' blackberry plants were placed in the dew chamber, the initial S.A. of leaves present at transfer (old leaf) was 2.70 µm. (Table 6). After the 1 M NaCl solution was applied the mean S.A. was 1.51 µm. The mean initial S.A. of the leaves tagged 2 weeks (week 2) after transfer to the dew chamber was 5.08 µm. After the NaCl solution was applied the S.A. was 5.31 µm. Only stomata on the leaves present at transfer were able to close in response to the application of the NaCl solution. Once the NaCl solution was replaced with buffer the S.A. of the "old leaf" was 1.60 µm and the "week 2" leaf 3.92 µm.

Stomata on these newly formed leaves resembled in vitro stomata in that a) they would not close in response to the 1 M NaCl solution although the protoplasm did collapse, and b) their initial S.A. was greater than on leaves developed under conditions of low relative humidity and high light intensity (in the greenhouse).

When these greenhouse-grown plants were placed under conditions of low humidity (25-30 %), leaves which developed in the dew chamber desiccated quickly despite the fact that they were growing on plants having a full root mass. This indicated that the plant could not balance the water lost through leaves with non-functional stomata by taking up greater amounts of water through the roots and subsequently increasing the transpiration rate through these leaves.

However, the plants had no problem controlling the transpiration rate in leaves which had originally developed in the greenhouse. This appears to contradict the idea that the lack of a functional root mass on transplants from culture is responsible for the lack of evapotranspiration control after transplantation (Debergh and Maene, 1981, Pierik, 1988). Stomatal function is impaired on leaves of blackberry which develop under conditions of high relative humidity and low light levels.

Figure 7. Photomicrographs of stomata of a 'Silvan' blackberry leaf, from plantlets grown on "Full MS", 3 days after removal from the dew chamber to the growth chamber. a) while on buffer; b) after application of the 1M NaCl solution; c) after replacement of the NaCl solution with buffer. Arrows indicate functional or partially functional stomata. Bar = 50 µm.


A
B
C

4.4. Conclusions:

Medium agar concentrations of 6, 9 or 12 g/l did not affect ex vitro survival or shoot height during the first 3 weeks ex vitro. However, plantlet height was reduced by medium agar concentrations of 9 and 12 g/l during the first 2 weeks after transplantation. By the third week plantlets from all medium agar concentrations were similar in height (2.84, 2.89 and 2.27 cm for 6, 9 and 12 g/l medium agar concentration respectively). Following transplantation, leaf number was reduced only when shoots were grown on the highest medium agar concentration (12 g/l). Plantlets consistently produced longer leaves ex vitro when compared to shoots. However, agar concentration had no observable effect on the longest leaf length. At the end of 3 weeks only plantlets from treatment 4 (plantlets grown on 9 g/l medium agar concentration) showed a significant relationship between initial and final fresh weight, this was linear (r^2 = 0.52). No relationship was found between initial and final fresh weight in treatments 2 and 6. Three weeks after transplantation, no relationship was found between persistent leaf number and final fresh weight of plantlets grown on any medium agar concentration.

Plantlets of 'Silvan' blackberry performed better than shoots during the first three weeks ex vitro. This was demonstrated by the greater transplant height, longest leaf length and the greater fresh weights in the rooted treatments. Rooting in vitro appears to override the initially negative aspects of firmer agar at a concentration of 12 g/l.

Despite the fact that the S.I. differed between persistent leaves and between weekly new leaf populations no particular trend could be found. No correlations were found between stomatal index and agar concentration or in vitro rooting.

A 1 M NaCl solution followed by replacement by buffer was found to

be the test of choice for studying stomatal function (closure and opening). Stomata of greenhouse-grown leaves exposed to the 1 M NaCl solution had a much shorter closure time (1-2 min) when exposed to a 2:1 ratio of 10^{-2} ABA/ buffer or a dark interval of between 25 to 35 min. Some difficulties were encountered when trying to photograph the epidermal peels after the NaCl solution was applied. It was sometimes hard to focus since the NaCl solution caused the removal of water from the cells and made the surface area uneven. The S.A. which resulted when the NaCl solution was replaced by buffer could be unreliable due to problems controlling the quantity of salt removed from the cells and the speed at which it was removed.

In response to the hypertonic solution (1 M NaCl), guard cell closure on epidermal peels improved in each successive new (weekly) leaf population (experiment 2) or new leaf (experiment 3) following transplantation. Stomatal function was therefore transitional. That is, some but not all stomata were functional during the first few weeks ex vitro, indicating that a change in environmental parameters during stomatal development may have influenced the ability of stomata to close. In experiment 2 stomatal function was observed 3 weeks after transplantation. In experiment 3 stomatal function was observed 2 weeks after transplantation. However, it was not until 3 weeks after transplantation that the majority of stomata from new leaves could completely close.

Decreasing the relative humidity from 95 % to approx. 50 % 2 weeks after transplantation, caused stomatal closure. Growing the plantlets on "1/4 MS" medium did not improve ex vitro stomatal function over growing plantlets on "Full MS" media. The relative humidity level had greater effect on stomatal closure than did the medium on which the plants were originally grown. The initial stomatal aperture of persistent leaves from both medium treatments was reduced when placed in the growth chamber for 1 week (following 2 weeks

in the dew chamber). Plant exposure to continued high relative humidity 2 weeks after transplantation appeared to reduce stomatal closure.

Stomata of new leaves of greenhouse-grown 'Silvan' blackberry plants that developed 2 weeks after transfer to conditions of high relative humidity and low light intensity (dew chamber) did not close in response to the application of the hypertonic solution. Stomata on original leaves (on the plants at transfer) closed in response to the NaCl solution after 4 weeks in the dew chamber. These results indicated that impaired stomatal closure in vitro may be in part due to environmental conditions such as high humidity levels in the culture environment.

During application of the NaCl solution it was observed that the guard cell protoplasm of non-functional stomata collapsed even though the guard cell aperture was not reduced. This indicated that the guard cell membrane was permeable and that modified anatomical properties of the guard cell walls were most likely responsible for the impaired stomatal function observed in cultured plant leaves and leaves from greenhouse-grown plants which had developed under conditions of high relative humidity.

5. CONCLUSION

A rapid clearing and staining method for tissue-cultured plantlets and greenhouse-grown leaves was successfully developed for the purpose of observing stomatal distribution on leaves of varying thickness. This method enables examination of large numbers of samples of many leaf types as long as they do not exceed 3 mm in width. A maximum of 2 hr are required to complete the clearing process. It is particularly useful for the observation of tissue-cultured shoots and plantlets since the whole plantlet can be cleared without detaching the stem or roots from the leaves. Disadvantages of the method include the killing of the cells during the process and the possible difficulty of focusing on large samples of uneven thickness.

In vitro 'Silvan' blackberry plantlets did not survive better after transplantation when grown on increased medium agar concentrations (9 and 12 g/l) compared with the control agar concentration (6 g/l). During the first 2 weeks ex vitro height was reduced in plants from medium agar concentrations over 9 g/l. Leaf number and length were not affected by medium agar concentration. Plantlets were generally taller and had longer leaves and a greater fresh weight when compared with shoots during the first weeks ex vitro suggesting that in vitro roots are functional ex vitro. Three weeks after transplantation, a linear relationship ($r^2 = 0.52$) was found between initial fresh weight and final fresh weight in treatment 4 (plantlets grown on 9 g/l medium agar). However, treatments 2 (plantlets grown on 6 g/l medium agar) and 6 (plantlets grown on 12 g/l medium agar) showed no relationship between initial fresh weight and final fresh weight. No relationship was found between initial leaf number and final fresh weight 3 weeks after transplantation for any treatment. Stomatal index was not affected by medium agar concentration or in vitro rooting. The stomatal

index of persistent leaves and all new leaf populations was greater than that of greenhouse-grown 'Silvan'.

Stomatal opening and closure in response to a 1 M NaCl solution was transitional in that stomata from each new leaf which developed ex vitro showed greater potential to close. After 2 weeks in a dew chamber, stomata on leaves of ex vitro plantlets from 1/4 strength rooting medium showed a reduced capacity to close when compared to those from full strength rooting medium. However, after this point there was no evident difference in stomatal function between the two treatments. Three weeks after transplanting, 'Silvan' plants placed in the growth chamber at the end of the second week, had stomata with a greater capacity to close in response to the NaCl solution than plants which remained in the dew chamber. The initial stomatal aperture of persistent leaves was smaller on plants placed in the growth chamber than those left in the dew chamber and only these stomata (in the growth chamber) were able to completely close ($\leq$ 2 µm). The capacity for stomata to reopen after the NaCl solution remained generally unaltered. Therefore, reduced relative humidity appeared to promote stomatal closure.

Three weeks after transplantation, stomata on new leaves of 'Silvan' plantlets placed under reduced relative humidity (55 %) for 1 week were functional. Stomata on leaves of 'Silvan' plantlets maintained under high relative humidity levels (100 %) for 3 weeks could not close completely. Protoplasm collapse was observed in response to the NaCl solution even if guard cells did not close indicating that stomatal non-function in vitro is most likely due to anatomical not physiological abnormality. New leaves that developed on greenhouse-grown plants after they had been transferred to a dew chamber had stomata which could not close in response to a 1 M NaCl solution. Thus high relative humidity levels and low light intensity may be largely responsible for impaired

stomatal function in culture.

In view of these results it would be unwise to raise in vitro medium agar concentrations above 9 g/l for the purposes of improving the time for and success of acclimatization. In vitro rooting of 'Silvan' blackberry appears to be a beneficial acclimatization strategy. Based on results from direct observations of stomatal function - blackberry plantlets should be maintained under conditions of high relative humidity for 2 weeks after transplantation from which point they should be transferred to conditions of lower relative humidity. The lower relative humidity levels will promote stomatal function thereby decreasing the time necessary for transfer to the field or greenhouse.

6. Suggestions for further research

1. Further experiments to determine the relationship between initial fresh weight and final fresh weight after transplantation would help optimize ex vitro transplant size.

2. Further study of the effect of high relative humidity and low light levels on greenhouse-grown plants would increase our understanding of the effects of these parameters on in vitro plant anatomy.

3. To determine why stomatal function is impaired under conditions of high relative humidity, it is necessary to complete anatomical studies focusing on the ultrastructure of the guard cell membranes and determining differences in structures which may be related to environmental conditions.

4. In vitro and ex vitro root function should be evaluated. This could be done using dyes or radioactive tracers and anatomical observations.

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APPENDIX I

Modified Murashige and Skoog's (MS)(1962)

basal medium with additions

Constituents	Full strength (mg/l)	1/4 strength (mg/l)
NH_4NO_3	1650.000	412.500
KNO_3	1900.000	475.000
H_3BO_3	6.200	1.550
KH_2PO_4	170.000	42.500
KIO	830.000	207.500
$Na_2MoO_4 . H_2O$	0.250	0.063
$CoCl_2 . 6H_2O$	0.025	0.006
$CaCl_2 . 2H_2O$	440.000	110.000
$MgSO_4 . 7H_2O$	370.000	92.500
$MnSO_4 . H_2O$	22.300	5.575
$ZnSO_4 . 7H_2O$	8.600	2.150
$CuSO_4 . 5H_2O$	0.025	0.006
EDTA - Ferric salt	43.000	10.750
Thiamine . HCl	0.400	0.100
Nicotinic acid	0.500	0.125
Pyridoxine . HCl	0.500	0.125
Glycine	2.000	0.500
Myo-inositol	100.000	25.000
Sucrose	30000.000	7.500
Growth regulators		
Multiplication medium		Rooting medium
BAP	1.00	BAP 0.10
IBA	0.10	IBA 0.50
Agar (Difco-bacto®)	6000.00	
pH	5.80	