DISSERTATION

PHYSIOLOGICAL STUDIES IN ACCLIMATIZATION OF IN VITRO TOBACCO PLANTLETS

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY FARIDA SAFADI ENTITLED **"PHYSIOLOGICAL STUDIES IN ACCLIMATIZATION OF IN VITRO TOBACCO PLANTLETS"**, BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PHYSIOLOGICAL STUDIES IN ACCLIMATIZATION OF IN VITRO TOBACCO PLANTLETS

Two acclimatization methods of in vitro tobacco (*Nicotiana tabaccum* 'Wisconsin 38') plantlets (IVP) were studied for their physiological effects upon plantlet response to transplanting: 1) the use of osmotica in rooting medium such as polyethylene glycol (PEG) or salts ($NaCl + CaCl_2$) at different concentrations and durations, and 2) the use of semipermeable closures (SPC) which improves gas permeability of culture vessels.

Concentrations of 1.0%, 2.5%, 5.0%, 10.0% and 15.0% PEG reduced water loss of detached leaves but affected growth adversely especially when above [5%] and 6-day-duration. PEG treatments induced epicuticular wax (EW) build-up and were related to reduced rates of water loss.

Salt treatments reduced water loss at 1.5 and 2.0% over control but caused undesirable growth characteristics.

Leaf diffusive resistance of PEG-treated plants was reduced prior to transplanting and remained lower than that of control. Stomates of IVPs had slower response to reduced humidity and darkness than greenhouse and PEG-treated plants.

The SPC-treated cultures had 2.5 times more evapotranspiration, 2% less relative humidity (RH) and 3 times less medium and plantlet leaf water potential than B-cap-treated cultures.

SPC treatments increased plantlet EW by 35%, reduced water loss by 60%, increased plantlet dry weights, reduced wilting injury and increased initial relative growth rates as compared to B-cap treatments.

Photosynthetic rates of in vitro plantlets were reduced at RH lower than 80-90%. SPC improved photosynthetic rates under desiccating conditions by reducing initial conductance and transpiration. Photosynthetic rates of IVPs from both closure treatments were comparable to those of greenhouse plants at high humidity. Stomates of plantlets from both treatments did not respond to [CO_2] and darkness compared to stomatal responses of greenhouse plants.

Chlorophyll content was increased in the SPC over B-cap plantlets. Better gas exchange of SPC was observed as indicated by CO_2 accumulation.

Although both osmotica and closure treatments reduced moisture loss in detached leaves of IVPs to levels comparable to those of greenhouse plants, the moisture loss curves deviated from normal bi-phasal shape indicating lack of normal stomatal functioning.

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This dissertation is dedicated

to my country Jordan

with Love and

Longing

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CHAPTER I

INTRODUCTION

The commercial mass propagation through tissue culture has become increasingly important for many plant species. However, the potential use of tissue culture plantlets is still limited by their poor acclimatization to ex vitro conditions. In vitro plantlets (IVP) of many plant species often exhibit low survival rates upon transfer to the external environment. Excessive water loss and inability of plantlets to switch to autotrophic nutrition are major factors causing death after transplanting to the greenhouse. The classical acclimatization methods of misting, humidity chambers and gradual increase in light intensity were time consuming and costly.

In vitro environmental conditions of high relative humidity (RH), low light intensity and gas exchange are known to confer abnormal physiological and anatomical transformations which are not adaptable to ex vitro conditions. Poor development of leaf surface wax, lack of stomatal functioning, abnormal internal leaf anatomy and poor root-shoot vascular connections have been described in tissue cultured plantlets. Plantlets with these features exhibited poor control over water loss upon transplanting, with consequent desiccation. The high water status of the tissue cultures was found to be responsible for most of the abnormalities in IVPs.

Acclimatization methods which included in vitro manipulation of water status (RH of the gaseous atmosphere and medium water potential) of the cultures have been successful in many plant species. Modification of water status in the culture vessels, however, is a delicate technique and requires thorough examination to maintain balanced and normal water relations in plantlets.

The photosynthetic system of in vitro plantlets has been reported to be either impaired or inactive due to the limiting in vitro conditions. However, plantlets with chlorophyll were found to have normal photosynthetic ability which is incapacitated by the restricted environmental conditions in vitro. The low CO_2 concentrations in the culture vessels, low light intensity and the presence of organic carbon source forced plantlets to be obligatory heterotrophic or mixotrophic during most of their culture period. Correcting in vitro environmental conditions by CO_2 enrichment, gas permeable closures, high light intensity, and sucrose omission enhanced photosynthesis and resulted in autotrophic growth in vitro. Growth and plantlet vigor were significantly promoted and the method was reported to have a potential in improving acclimatization of IVPs.

In this research two methods of acclimatization of in vitro tobacco plantlets were tested: 1) modification of water status of the culture by decreasing water potential of the medium using polyethylene glycol (PEG) or salts ($NaCl+CaCl_2$) in vitro prior to transplanting and 2) increasing gas exchange of in vitro cultures by using semipermeable plastic film closures (SPC).

The first treatment is expected to precondition the plantlets to water stress and induce features that enhance normal control over water loss. Therefore,

features such as water loss of detached leaves, epicuticular wax content and leaf diffusion resistance were examined in the first two papers: Chapters 3 and 4 of this dissertation.

The second treatment (gas permeable closures) is presumed to change water status of the cultures through reducing the relative humidity of the gaseous atmosphere in the vicinity of the plantlets. In turn, it is expected to modify the photosynthetic activity and leaf conductance of the plantlets by improving gas exchange, such as CO_2 and water vapor in the vessels. Therefore two studies were made with this treatment: 1) a study which examines water relations of plantlets grown under SPC treatments, including their water potentials, water loss, wax content and acclimatization in the greenhouse (Chapter 5), and 2) a study which examines the photosynthetic activity and leaf conductance of in vitro tobacco plantlets in responses to reduced RH after transplanting, $[CO_2]$ and darkness (Chapter 6). This study would reveal plantlet acclimatization competence in terms of photosynthetic ability and control over water loss by stomatal conductivity.

Under all conditions, growth of plantlets in vitro is examined to study the effect of acclimatization treatments on plantlet development. Tobacco (*Nicotiana tabaccum* L. "Wisconsin 38") was chosen as a representative herbaceous plant which is relatively sensitive to transplanting. Tobacco plants are a model for many physiological studies in plant tissue cultures.

The general objective of these investigations was to study some of the physiological changes that accompany the acclimatization treatments used: osmotica

and the gas permeable closures and their effect upon acclimatization of tobacco plantlets.

Specifically the study was carried with four objectives:

1) To evaluate the effect of PEG treatment on moisture loss, wax content, and growth of tobacco plantlets as compared to non-treated and greenhouse plants.

2) To examine the effect of PEG on stomatal function of tobacco plantlets through porometric studies of leaf diffusive resistance and anatomical observations of stomates after transfer ex vitro.

3) To evaluate the effect of the gas permeable closure (SPC) on water relations of tobacco cultures including the evaporative water loss of the vessels and their relative humidity, medium and plant water potential, plantlet water loss, wax content and their growth and survival upon transfer to the greenhouse.

4) To evaluate the effect of SPC on photosynthesis and leaf conductance of tobacco plantlets in response to reducing levels of RH, low and high CO_2 concentrations and darkness.

It is hoped that these studies would enable better understanding of the physiological effects of the acclimatization methods used and help the choice for appropriate procedures for acclimatizing IVPs.

CHAPTER II

LITERATURE REVIEW

A. General Background

Plant tissue culture as a means of propagation (micropropagation) has been shown to be a viable alternative to the traditional propagation of many plant species. Micropropagation has provided the industry with several advantages including: mass propagation of theoretically identical plants, the ability to propagate plant species that are difficult to propagate by conventional methods, the production of virus-free, healthy and vigorous plants, and several usages in plant breeding and crop improvement (Murashige, 1974; Grout and Crisp, 1977; Zilis *et al.*, 1979; Clare and Collins, 1974).

The protocol of micropropagation may be divided into four major stages (Murashige, 1974). These stages are:

Stage I: establishment and initiation of the aseptic culture;

Stage II: induction of growth and multiplication of propagules;

Stage III: preparation for transfer to soil; includes rooting and acclimatization (in vitro hardening); and

Stage IV: transfer to ex vitro conditions and establishment in soil; also includes acclimatization.

Considerable research has been done on optimizing conditions for growth and multiplication, stages I and II. However, relatively less attention has been given to the conditions needed for acclimatization and establishment in soil (Murashige, 1974; Zilis *et al.*, 1979; Skolmen and Mapes, 1978; Wetzstein and Sommer, 1983). Acclimatization of plants in stage III as described by Murashige (1974) depended upon the reduction of nutrient levels in the medium, supplementation of the medium with root promoting hormones and culture under elevated levels of irradiance. Acclimatization in the greenhouse included the gradual exposure to external conditions of humidity and light intensity (Murashige, 1974). However, these measures were inadequate to enhance survival after transplanting (Ziv, 1986). Poor establishment of propagules in soil due to inadequate hardening procedures, has continued to be a major hindrance for large scale adaptation of the micropropagation technology by industry (Broome and Zimmerman. 1978; Earle and Langhans, 1974; Vertezy and Balla, 1987; Conner and Thomas, 1981; Sutter and Langhans 1979; Skolmen and Mapes, 1978; Pierik, 1988). In many plant species, losses from 50 to 90 % of in vitro propagated plantlets were encountered at the time of transfer to soil thus making the operation costly and unfeasible (Sutter and Langhans, 1979; Grout and Crisp, 1977; Hasegaswa *et al.*, 1973; Donnan *et al.*, 1978; Fuchigami *et al.*, 1981; Ziv *et al.*, 1983). Accordingly, the ultimate success of tissue culture as a commercial means of plant propagation depends upon adequate acclimatization and successful establishment of plantlets in soil (Conner and Thomas, 1981; Short *et al.*, 1987; Sutter and Hutzell, 1984).

In a series of studies, the lack of acclimatization of in vitro plantlets (IVPs) was related to a specialized physiological abnormality of in vitro cultures called 'vitrification'. Vitrification was frequently observed in several plant species and was shown to be a major limitation for mass propagation of plants (Debergh *et al.*, 1981; Bornman and Vogelmann, 1984; Ziv *et al.*, 1983; Kevers *et al.*, 1984; Paques and Boxus, 1987; Arnold and Eriksson, 1984). Vitrification also referred to as glassiness, translucency, vitrescence, and hyperhydric malformations, was described as a physiological disorder in microplants characterized by abnormal leaf development, broad, thick, translucent, wrinkled, low in chlorophyll and easily breakable leaves (Debergh *et al.*, 1981; Paques and Boxus, 1987; Phan and Hegedus, 1986). Vitrified leaves lacked palisade tissue, containing only spongy mesophyll with lacunal space filled with water (Leshem, 1983a; Arnold and Eriksson, 1984). Defective cellulose and lignification of the vessels and tracheids in vitreous plants was also recognized (Kevers *et al.*, 1984). This abnormal anatomy was, to variable degrees, typical of in vitro plants noted for their lack of acclimatization (Wetsztein and Sommer, 1982; Brainerd *et al.*, 1981; Grout and Aston, 1978a; Donnelly and Vidaver, 1984a). The term 'vitrification' was first used to describe the physiological disorder responsible for the vitrified aspect, i.e. decreased proliferation and necrosis of tissue cultures. However, since the problems encountered with the transfer of plantlets to soil in situ were also considered a manifestation of vitrification, the term was also used to denote lack of acclimatization of IVPs (Paques and Boxus, 1987; Gaspar *et al.*, 1987). Indeed, it is considered by some as a severe case of lack of acclimatization or an aleatory disease facing industrial tissue culturists (Paques

and Boxus, 1987; Leshem, 1983b; Ziv *et al.*, 1983). The multiplicity of factors reported to cause vitrification and the observed variety of its expressions caused its designation as a separate characteristic of in vitro culture (Leonhardt and Kandeler, 1987; Paques and Boxus, 1987). Plants, however, are known to vary in their transplantability from those which do not need any acclimatization treatment to those which require extreme care in transplanting (Bowden, 1984; Mckee, 1981; Conner and Thomas, 1981; Groutand Millam, 1988).

In later research, vitrification was studied in relation to problems associated with survival upon transplanting and hence will be incorporated in this review wherever pertinent (Ziv *et al.*, 1983; Singha *et al.*, 1990; Dillen and Buysens, 1989).

Establishment of plantlets ex vitro has remained a problem for many plant species, especially those exhibiting vitrification. A large body of information has been generated which described the nature of the abnormality that result in low survival of IVPs upon transfer to ex vitro conditions (Sutter and Langhans 1979; Conner and Conner, 1984; Wardle *et al.*, 1979; Grout and Aston, 1977a; 1977b; 1978; Brainerd *et al.*, 1981; Fuchigami *et al.*, 1981; Wetzstein and Sommer, 1982; 1983; Lee *et al.*, 1988; Donnelly *et al.*, 1984a; 1984b). This research related low IVP survival to in vitro environmental conditions which might be responsible for the abnormalities. Once these problems were identified, other methods of hardening were explored (Wetzstein and Sommer, 1982; Conner and Conner, 1984; Pospisilova *et al.*, 1988; Fujiwara *et al.*, 1988).

B. Problems Associated With Transplanting IVPs to Ex Vitro Conditions

I. Introduction

When tissue culture plantlets are transferred to ex vitro conditions, they sustain excessive and uncontrolled water loss. This results in desiccation and transplanting shock of IVPs (Brainerd and Fuchigami, 1981; Grout and Aston, 1977; Sutter and Langhans, 1979; Vertesy and Balla, 1987). Furthermore, the low survival of IVPs has been related to the transition from heterotrophic to autotrophic nutrition (Grout and Aston, 1978; Wetzstein and Sommer, 1982; Donnelly *et al.*, 1984b). Poor control over water loss and defective photosynthesis (Ps) are two main deficiencies of micropropagated plantlets which render them vulnerable to transplantation shock (Wetzstein and Sommer, 1982; Ziv, 1986)

II. Water Loss of In Vitro Plantlets

a. Description. A considerably higher rate of water loss from excised leaves of tissue-cultured plantlets than that for plants in greenhouse has been demonstrated in *Malus domestica* (Borkh.) cv. Mac 9 (Brainerd and Fuchigami, 1981), *Prunus institia* L. cv. Pixie (Brainerd *et al.*, 1981), *Solanum laciniatum* Ait (Conner and Conner, 1984), and *Leucaena leucocephala* (Lam.) De Wit cv. K-8 (Dhawan and Bhojwani, 1987). Higher transpiration rates were found for tissue-cultured *Brassica oleracea* var. *botrytis* plantlets compared to seedlings (Grout and Aston, 1977; Wardle *et al.*, 1979; 1983a) as well. In these experiments, more than 50% of the leaves' moisture content was lost within 30 minutes for *P. institia* and *L. leucocephala*, and 70 minutes for *S. laciniatum*, compared to 140, 90 and 140 minutes for greenhouse plant leaves respectively. Electrolyte leakage and

ethylene/ethane analyses have indicated cell injury in *P. institia* leaves at 50% water loss (Kobayashi *et al.*, 1981). Thus excessive water loss upon transplanting to ex vitro conditions has been described as due to poor control over water loss by the leaves, and possibly due to inadequate water uptake resulting in unbalanced water relations (Grout and Aston, 1977; Wardle *et al.*, 1979; Sutter, and Langhans, 1979; Sutter, 1988; Conner and Thomas, 1981). Most attention has been directed towards the role of leaves in the rapid water loss (Sutter, 1985).

b. Physiological and Anatomical Features Associated With Water Loss of Tissue Culture Plantlets

1. Leaf Epicuticular Waxes

a) In Vitro Plants. The first leaf characteristic associated with excessive water loss was the lack of epicuticular wax (EW). Grout and Aston (1975; 1977) reported that leaves from in vitro *B. oleracea* lacked EW as observed by scanning electron microscope (SEM) and contact angle when compared to greenhouse seedlings and acclimatized plantlets. SEM wax observations were correlated with amounts of waxes and increased cuticular water loss in IVPs over that of seedlings (Grout and Aston 1975; 1977).

In other SEM studies, lack of structural EW formation (or wax bloom) on leaf surfaces of in vitro carnations (*Dianthus caryophyllus* L. 'White Pike's Peak'; Sutter and Langhans, 1979) and cauliflower (*Brassica oleracea* var. *Capitata* 'Market Prize'; Sutter and Langhans, 1982) was confirmed and correlated with non-glaucousness, low amounts of wax, increased water loss and low survival of IVPs. The morphological structure of EWs was further correlated with amounts and

composition of wax (Sutter, 1984). Since permeability of plant cuticles to water have been shown to be affected by composition and components of wax which affected wax morphology (Haas and Schonerr, 1979; Possingham *et al.*, 1967; Schonerr *et al.*, 1984; Grncarevic and Radler, 1967), the unrestricted water loss in in vitro plants was attributed to the lack of structural EWs (Sutter and Langhans, 1982).

The studies of Grout and Aston (1975, 1977a) and Wardle *et al.* (1979) have detected an early closure of stomates (within 5 to 30 minutes after removal from culture), thus, wax appearances and contents were thought to be specific to cuticular water loss. They concluded that in the absence of cuticular waxes the stomates of IVPs were unable to control water loss sufficiently to maintain turgor and prevent wilting. However, these studies did not take into consideration the amount of water lost due to open stomates in the first five to thirty minutes, and their impact upon wilting. They also did not describe their experimental decision of stomatal closure (Brainerd and Fuchigami, 1981).

Later studies underscored the importance of EW in water conservation of IVPs since the previous studies by Sutter and Langhans (1979; 1982) could not be extended to all plant species (Sutter, 1985). Some plantlets were found to have amorphous smooth wax deposition on leaf surfaces, or possibly intracuticular waxes which were not detected by SEM. SEM observations of *Chrysanthemum xmorifolium* Ramat. 'Bright Golden Anne' and *Spathiphyllum wallissii* plantlets revealed smooth surfaces, yet in vitro chrysanthemum had 10 times as much wax content as *Spathiphyllum* and survived better upon transfer. Cultured plants of

Spathiphyllum had twice as much wax content as their greenhouse counterparts. In vitro carnations had smooth surfaces but contained more than twice the amount of wax as *Maranta leuconeura* although the latter appeared to have dense structural wax. *Maranta* plantlets, however, had poor survival rates in the greenhouse (Sutter, 1985). No linear correlation was found between amounts of wax and survival of plantlets upon transfer (Sutter, 1985). Similarly, smooth non-structural EWs were observed on leaves of sweetgum (*Liquidambar styraciflua* L.) from greenhouse, growth chamber and in vitro (Wetzstein and Sommer, 1983), while transmission electron microscope observations revealed differences in cuticular thickness (Wetzstein and Sommer, 1983). Leaves of in vitro and greenhouse blackberry (*Rubus* sp. 'Silvan') had smooth surfaces; adaxial water losses were attributed to hydathodes rather than cuticular (Donnelly *et al.*, 1987). Leaves of in vitro roses (*Rosa multiflora* L. cv. Montse) were observed to have more crystalline wax deposition than those of the greenhouse-grown leaves (Capellades *et al.*, 1990). Thus, the morphology of EW as observed by SEM was found to be neither typical of the growth conditions of the plants, nor depicting the gravimetric amounts of wax on leaf surfaces or the survival ability of plantlets upon transfer. Sutter (1985) concluded, therefore, that SEM observations of wax structure, could not be used to determine the amounts of wax present and to predict the adaptability of tissue culture plants upon transfer. Quantitative analysis of leaf surface wax was shown to be necessary before any conclusions were made about cuticle permeability of in vitro plants based upon SEM (Sutter, 1985).

Other studies reported increased amounts of wax and observed structural wax using SEM, with acclimatization of tissue culture plantlets, but did not correlate them directly with water loss or survival. Wardle *et al.*, (1979) observed reduced wax deposits on leaves formed during culture, but increased surface wax on new leaves formed after transfer ex vitro. They also demonstrated that spraying an antitranspirant (polyvinyl resin S600), known not to affect stomata, increased the cuticular resistance of IVPs and eliminated their need for acclimatization. Thus, they concluded that poor survival of untreated plantlets was due to excessive cuticular transpiration (CT). However, the same authors also detected poor stomatal functioning in in vitro leaves and restoration of the function in newly formed leaves after transfer. They reconciled their findings by concluding that the acclimatization was due to the control of water loss through both cuticular wax and stomates.

Wardle *et al.* (1983a) reported increased amounts of EW and reduced water loss in acclimatized cauliflower (*B. oleracea*) plantlets. They also determined that stomatal closure was induced by the same acclimatization treatments. Short *et al.*, (1987) reported increased amounts of EW in *Chrysanthemum xmorifolium* Ram. 'Pennine Reel' and *B. oleracea* after eight weeks of hardening. Typical wax bloom was observed on leaves of *B. oleracea* with acclimatization treatments and was correlated with decreases in water loss and improved survival upon transfer to soil

Fari *et al.*, (1987) induced profuse wax excretion on the leaf surfaces of in vitro onions (*Allium cepa*) by low relative humidity (RH) hardening. They associated wax formation, amongst other features induced, with successful transfer

into soil. Dhawan and Bhojwani (1987) documented progressively increasing structural wax in in vitro *L. leucocephala* during their rooting/in vitro acclimatization period of 3 weeks, post acclimatization period of 4 weeks and field establishment. They also detected progressively decreasing water loss of detached leaves in the same above order (Dhawan and Bhojwani, 1987), but other factors were also found to affect water loss. Glaucousness, cuticular wax and improved survival as well as stomatal functioning were induced in carnation (*Dianthus caryophyllus* L. 'Ceris Royallete') plantlets hardened by reducing water status of the culture (Ziv *et al.*, 1983; 1987). Marin and Gella, (1988) found similar cuticular structure in in vitro and in vivo cherry (*Prunus cerasus* L. 'Masto De Montana') plants, but observed a thinner cuticle layer in in vitro leaves which lacked cuticular ridges. They described higher rates of water loss in IVPs than that of seedlings, but attributed it to the different anatomical features of in vitro plants as well as cuticle. They further showed that survival of cherry plantlets was not related to water loss since plant death occurred 15 days after transfer, after the critical period of water loss was overcome (Marin and Gella 1988).

Wetzstein and Sommer (1982) described cuticle structure of in vitro *L. styraciflua* plantlets to be similar to that of seedlings with a thinner cuticle of the cultured plantlets. However, water conductance via the cuticle was shown not to be directly related to its thickness (Jordan *et al.*, 1984; Schonherr, 1976).

Several studies have shown a less important role of EWs than stomates in water loss of plantlets (Brainerd and Fuchigami, 1981; Fuchigami *et al.*, 1981; Conner and Conner, 1984). Water loss of abaxial surfaces of greenhouse seedlings

and two-week-acclimatized plum (*P. institia*) was minimal although they contained no wax coverage. In contrast, the abaxial surfaces in in vitro plantlets exhibited excessive water loss (Fuchigami *et al.*, 1981), indicating other factors than wax, which control water loss. Adaxial water loss was insignificant in all treatments, although quantitave differences in wax occurred among treaments (Fuchigami *et al.*, 1981). This indicated no correlation between wax presence and water loss (Fuchigami *et al.*, 1981).

Since wax deposition took 10 days to 2 weeks in transferred *B. oleracea* (Grout and Aston, 1977; Wardle *et al.*, 1979) and 2.5 weeks in carnation (Sutter and Langhans, 1979) after transfer to the greenhouse, Brainerd and Fuchigami (1981) believed that acclimatization of plantlets and improved survival was not due to increased EW content. The long time needed for EW development did not obviate the need for urgent control of water loss upon removal from culture and failed to explain the successful hardening of in vitro apple (*M. domestica*) plantlets obtained in 4 days (Brainerd and Fuchigami, 1981).

In acclimatizing tobacco (*Nicotiana tabaccum* L. 'White Burley') plantlets, stomatal conductance was restored to normal levels after 3 weeks of acclimatization while cuticular conductance lagged behind indicating the slow production of EWs during acclimatization (Pospisilova *et al.*, 1987). Conner and Conner (1984) observed the absence of structured EW from in vitro *S. laciniatum*, but reported that leaf cuticles were only effective in controlling water loss after stomatal closure. They also observed little wax after acclimatization for one month. Wetzstein and

Sommer (1983) observed lack of epicuticular structural changes upon acclimatization of *L. styraciflua*.

Cuticles were reported to be important as a pathway for water loss only when stomates were closed (Wenkert, 1983). Cuticular resistances were ignored in studies of leaf resistances to water vapor since cuticular water loss was observed to be much smaller than the stomatal fluxes (Wenkert, 1983; Pearcy, 1983).

b) Nature and Function of Plant Epicuticular Waxes

Cuticles of plants are described as consisting of a cuticular matrix embedded in cuticular wax. The wax can be embedded in the cuticular matrix and sometimes protruded on the leaf surface forming EWs. Plants that contain structural deposits of wax on the leaf surface or 'wax bloom' are called glaucous. Glaucousness in plants and extracuticular waxes are reported to be genetically controlled and vary among plant species, varieties, and environmental conditions. The function of wax bloom in water loss control is controversial (Baker, 1974; Hall *et al.*, 1965; Bengston *et al.*, 1978; Denna, 1970; Schieferstein and Loomis, 1956; Daly, 1964).

Intracuticular waxes have been reported to have an important role in cuticular permeability to water (Schonherr, 1976, Hall, 1966; Svenningsson and Liljenberg, 1986; Schonherr and Schmidt, 1979). The resistance of cutin matrix to water loss was found to be negligible while most of the resistance was conferred by the cuticular wax component (Schonherr, 1976). Schieferstein and Loomis (1956), based upon their observation that many plants had no surface wax, believed that a continuous cuticular layer, possibly with subcuticular wax, rather than a surface bloom per se, was the major factor in water conservation. The report observed no

clear-cut relationship between surface wax and xeromorphic adaptation, so that the presence or absence of wax had doubtful survival value (Schieferstein and Loomis, 1956).

Denna (1970) did not detect a correlation between wax content within either glaucous or non-glaucous *Brassica oleracea* siblings, and water loss, and could not show clearly that wax bloom was the only factor which controlled cuticular water loss in glaucous leaves. Bengtson *et al.*, (1978) found no simple relationship between the reduction in CT and amounts of EW or changes in the chemical composition of EW which varied amongst six drought acclimated oat (*Avena sativa* L.) varieties, and concluded that there was another factor in addition to EW affecting CT. Similar results were obtained by Larsson and Svenningsson, (1986) in barley and by Svenningsson and Liljenberg (1986) in oats, where decreases in CT were correlated with chemical changes in the intracuticular waxes. Hunt and Baker (1982), could not find a consistent relationship between amounts of epicuticular wax and water loss among different species. The amounts of water lost by unregulated CT varied greatly with different types of plants, mainly due to differences in the chemical composition of waxes (hydrophobicity) and their effect on architecture of wax more than the amounts of epicuticular wax (Baker and Bukovac, 1971; Grncarevic and Radler, 1967; Possingham *et al.*, 1967; Schonherr *et al.*, 1984; Schonherr and Merida, 1981; Denna, 1970; Bengtson *et al.*, 1978)). For this reason and because of the inconsistent results amongst in vitro plant species, generalizations about the role of EW in water loss in in vitro plants should be made with caution (Sutter, 1985).

The consistent report of the lack of or low EWs in IVPs, may indicate that CT, although secondary to stomatal transpiration, contributes to the excessive water loss of plantlets upon transfer. It is possible that the presence of EW at time of transfer may help conserve some water while the stomatal function and other physiological functions are being restored. Bengston *et al.*, (1978) reported that any change leading to a reduction in water loss (reduction in CT) is advantageous during conditions of water stress. However, the fact that most studies of EW of IVPs were made on glaucous plants implied skepticism of the early conclusions where excessive water loss of IVPs were attributed to lack of structural EWs (Sutter, 1985).

However, other studies have emphasized the role of EWs in water loss control and adaptability to water stress or drought tolerance (Jordan *et al.*, 1983; 1984; Possinngham *et al.*, 1967; Hall, 1966; Bengston *et al.*, 1978; Daly, 1966). The importance of EW for CT has been indicated by the increase in CT obtained when EW was partially removed (Possingham *et al.*, 1967; Denna, 1970; Hall, 1966). After stomatal closure, leaf EWs were reported to be important in increasing the efficiency of stomatal control of water loss by reducing the cuticular conductance to water vapor (Jordan *et al.*, 1984; Schonerr and Schmidt, 1979; Bengtson *et al.*, 1978). Although the role of EWs in water loss is complex, it is concluded that EWs, if present through genetic constitution or by induction through environmental factors, serve an important but auxiliary role to that of stomates in controlling water loss of IVPs. Other plants which do not have genetic traits for epicuticular structural waxes or bloom, may contain an efficient, impervious, continuous layer

of cuticle with intracuticular waxes or other mechanisms for controlling water loss (Sutter and Langhans, 1979; Dhawan and Bojwani, 1987; Schefferstein and Loomis, 1956; Denna, 1970; Bengtson *et al.* 1978). This suggestion may reconcile the contention of Schefferstein and Loomis (1956) and the findings of Denna (1970) and Bengtson *et al.*, (1978) of no correlation between EW and water loss with those who suggest an important role of EWs in water conservation. It also may explain the inconsistent findings found by Sutter (1985) among different in vitro plant species relative to the importance of waxes and their water loss.

c) Effect of Environmental Factors on Epicuticular Wax Formation

The reason for the lack of structured epicuticular wax on IVPs remains unknown (Sutter and Langhans, 1979). However, the environmental factors under which plants grow can modify the morphology and the chemical composition significantly (Sutter and Langhans, 1979; Wetzstein and Sommer, 1982). Wax density and dimensions are affected by humidity, radiant energy, and temperature (Baker, 1974; Hallam, 1970; Juniper, 1960; Martin and Juniper, 1970; Whitecross and Armstrong, 1972). High humidity, high temperature and low light intensity, all conditions of tissue culture, decreased epicuticular wax formation in both brussels sprout (*Brassica oleracea* var. gemmifera; Baker, 1974) and field rape (*Brassica napus* L.; Whitecross and Armstrong, 1972).

Environmental conditions affected chemical composition of wax as well as quantity. It is known that the chemical composition of EWs is as important as their density in reducing leaf transpiration (Hadley, 1980; Crncarevic and Radler, 1967; Baker and Bukovac, 1971). Baker (1974) reported that in *B. oleracea*, higher

temperatures resulted in a lower alkane content and a higher aldehyde content. Giese (1975) found that in barley, light and temperature strongly influenced the chain length composition of the wax. Bengtson *et al.* (1978) observed changes in primary alcohols and alkane contents of epicuticular waxes in water stressed oats. They suggested that such changes have an impact on the EW structure which in turn affect water loss.

The relationship between reduced EW and high humidity has been documented (Watson, 1942a; Baker, 1974; Dale, 1982) in in vivo plants. Vapor pressure deficits (VPD) are a major factor affecting the physical properties of wax deposits (Hunt and Baker, 1982). Reduced VPD decreased both the complexity and amount of epicuticular wax on cabbage plants grown under high RH as compared to those grown under lower RH (Baker, 1974; Sutter and Langhans, 1982). The effect of low RH in stimulating wax production is consistent with the increase in wax deposits of plants of *Poa colensoi* under arid conditions compared with those grown in wet habitats (Daly, 1964; Dale, 1982). Bengtson *et al.* (1978) suggested that increased RH may cause decreased epicuticular wax formation through its effect on the water status of the plant. Increased EW production occurred in oat (*A. sativa*) plants subjected to water stress (Bengtson *et al.*, 1978).

Light influence on leaf EW formation was observed in differences in waxiness of sun and shade leaves (Esau, 1977; Skoss, 1955; Dale, 1982; Salisbury and Ross, 1985; Whitecross and Armstrong, 1972) where leaves growing in the sun had significantly higher wax content. Reed *et al.* (1982) observed significant increases in wax contents and changes in wax structure of carnation (*D.*

caryophyllus) and brussel sprouts (*B. oleracea* L., *Gemmifera* group 'Jade Cross E') with increasing light intensity. As light intensity decreased, EW rod length decreased and density increased. Lee *et al.* (1988) however, could not induce EW formation by increasing light intensity in in vitro *L. styraciflua* species.

The inevitable environmental conditions of high humidity and low light intensity appear to be responsible for the decreased amounts of wax formed on plants grown in vitro (Sutter and Langhans, 1982). Grout (1975), Grout and Aston (1977), Sutter and Langhans (1979), Sutter and Langhans (1982) as well as Conner and Conner (1984) observed differences in amount of epicuticular wax and relative wax morphology between plants grown in vitro and those grown in a growth chamber or in a greenhouse. In addition, wax of cultured plants had a different complexity of its crystalline structure compared with that in the greenhouse (Sutter and Langhans, 1982; Sutter 1984). Since wax complexity is an indication of a change in chemical composition, the in vitro environmental conditions had modified the components of wax which in turn affects water loss of in vitro leaves (Sutter and Langhans, 1982; Sutter, 1984).

Grout, (1975) and Grout and Aston (1977) concluded that the high RH in culture was the main cause for reduced epicuticular waxes since seedlings grown under the same environmental conditions of tissue culture developed comparable EW to that of in vitro plants. Moreover, seedlings grown, un-enclosed, under the same environmental conditions developed more waxes than in vitro plants (Grout and Aston, 1977; Sutter and Langhans, 1982). More direct evidence was obtained from the significant increase in EW detected upon reduction of the relative

humidity in the culture vessel from 100% to 35%. Other studies also correlated wax deposition with RH since wax deposition was increased consistently with acclimatization treatments of reducing RH (Grout and Aston, 1977; Wardle *et al.*, 1983a; Short *et al.*, 1987; Wardle *et al.*, 1979; Marin and Gella, 1988; Fari *et al.*, 1987; Ziv *et al.*, 1983; Smith *et al.*, 1990a; Capellades *et al.*, 1990).

Water stress of the culture medium has also been found to cause increased EW formation quantitatively and structurally through the use of increased agar concentration (Ziv *et al.*, 1983) or polyethylene glycol (Short *et al.*, 1987; Zaid and Hughes, 1990; Safadi and Hughes, 1992; Dami, 1991). Plants grown in liquid culture had reduced amounts of wax as compared to those planted on solid culture (Ziv *et al.*, 1983; Wardle *et al.*, 1983a). Sutter and Langhans (1982) suggested that the effect of reduced RH on increasing EW was through water stressing the plants by medium drying. Water stress was a factor which causes heavier cutinization of leaves (Skoss, 1955). Leaf waxiness of sun leaves was attributed to the effect of the sun exposure on water relations of the plants (Skoss, 1955). Daly (1964) indicated that waxiness of leaves is an adaptation to arid climate and that it has a physiological significance in reducing transpiration of leaves under hot and arid conditions. Bengston *et al.* (1978) observed increased amounts of wax and changes in wax composition in six oat varieties after long water stress periods. However, Hunt and Baker (1982) could not differentiate between the effect of atmospheric aridity and soil water stress on wax formation. While they detected increased wax formation in some xerophytic plants, they found other desert plants which lacked

wax deposits. When two lines of geranium plants were subjected to water stress, one developed a thicker cuticle while the other did not (Hunt and Baker, 1982).

The low light intensity in culture also appeared to influence EW of in vitro plants. However, little wax was observed on non-glaucous in vitro-grown leaves of carnation (*D. caryophyllus*) upon exposure to high light intensities (10 Klx) (Sutter and Langhans, 1979). Light intensities of 80 $\mu mol.s^{-1}.m^{-2}$ increased EWs in *R. multiflora* but the treatment was combined with 75% RH (Capellades *et al.*, 1990). The effect of light intensity on wax formation could be realized indirectly in studies reporting increased EW upon acclimatization ex vitro and from differences in EW between in vitro and greenhouse plants (Grout and Aston, 1977; Sutter and Langhans, 1979; 1982; Conner and Conner, 1984). Other light intensity studies concentrated on the effect of increasing light intensity on internal anatomy and Ps of the leaves (Donnelly and Vidaver, 1984a; 1984b; Desjardins *et al.* 1987a; 1988, Lee *et al.*, 1988; Kozai *et al.*, 1988a; Kozai *et al.*, 1990b). In all of the above studies, increased light intensity improved the examined characteristics relating to acclimatization of IVPs (Donnelly and Vidaver, 1984a; 1984b; Desjardins *et al.*, 1987a; 1988, Lee *et al.*, 1988; Kozai *et al.*, 1988a; Kozai *et al.*, 1990b).

No studies have been made of the effect of temperature on EW formation in IVP. However, the relatively high temperature (25 to 27°C) of in vitro cultures may not encourage wax formation (Sutter and Langhans, 1979). Whitecross and Armstrong (1972) reported the most reduced wax quantities in *B. napus* at day/night temperatures of 27/22°C as compared to lower temperatures of 21/16°C. High temperatures of 28°C caused changes in the chemical composition of EW on

tobacco (*Nicotiana tabaccum* L. cv. Hicks) leaf surfaces (Wilkinson and Kasperbauer, 1972).

The studies of Grout and Aston (1977) and Sutter and Langhans (1982) which compared in vitro plantlets with their open-grown-seedling counterparts incubated in the same growth chamber, reported that the only difference between the two treatments was the RH. However, these studies ignored the effect of the limited gas exchange and low CO_2 in the closed vessel of in vitro cultures. In comparison, seedlings grew under the more abundant CO2 in the growth chamber whereas IVPs grew unnder limited CO_2 concentrations in the vessels. Low CO_2 concentrations in the culture vessels have been documented and their effect on reducing on photosynthesis and dry matter accumulation was shown (Fujiwara *et al.*, 1987). Wax deposition was suggested to be related to the dry matter accumulation which requires efficient photosynthesis (Lakso *et al.*, 1986).

2. Stomatal Function

a) Stomates In In Vitro Plantlets

Stomates are the major opening of the plant to the atmosphere and control gas exchange in plants (Jarvis and Morison, 1981). Therefore, stomates have been studied relative to their role in water vapor loss and desiccation of transplanted tissue cultured plants.

Stomates of in vitro apple (*M. domestica*) were found to remain open for the one hour after plantlets were transferred to ex vitro conditions (Brainerd and Fuchigami, 1981). The rate of water loss from their excised apple leaves was linearly related to stomatal closure (Brainerd and Fuchigami, 1981).

Water loss of in vitro plum (*P. institia*) was shown to be mostly from abaxial surfaces where all stomates occurred (Fuchigami *et al.*, 1981). Likewise, water loss experiments with coated leaf surfaces of blackberry (*Rubus* sp.) indicated that all water loss took place through open stomates on the abaxial surface or hydathodes on the adaxial surface but not through the cuticle (Donnelly *et al.*, 1987). Acclimatization of in vitro apple (*M. domestica*) plantlets (Brainerd and Fuchigami 1981; 1982), *B. oleracea* (Wardle *et al.*, 1979), *C. xmorifolium*; Wardle *et al.*, 1983a; Smith *et al.*, 1990a; 1990b), carnation (*D. caryophullus*; Ziv *et al.*, 1987), cherry (*P. cerasus*; Marin and Gella, 1988) and roses (*R. multiflorum*; Capellades *et al.*, 1990) was associated with the restoration of stomatal closure.

Wardle *et al.*, (1979) observed that stomatal resistance of in vitro *B. oleracea* did not increase in response to ABA applications while those of seedlings did increase with ABA treatment. Stomates of in vitro apples (*M. domestica*) also failed to respond to closure stimuli such as mannitol induced water stress, ABA, darkness, and increased CO_2 concentrations (Brainerd and Fuchigami, 1982). Similarly, stomates of in vitro chrysanthemum did not close with exposure to darkness, ABA, and high levels of CO_2 whereas further opening was induced by CO2-free air and kinetin (Wardle and Short, 1983). Ziv *et al.*, (1987) could not induce stomatal closure in in vitro carnation *(D. caryophyllus)* in response to darkness, ABA or Ca^{++}, all of which are known to cause closure in functional stomates. Leaf stomates from in vitro blackberry (Donnelly *et al.*, 1986) and from in vitro sweetgum (*L. styraciflua*) did not close at night (Wetzstein and Sommer, 1983; 1988).

Microscopic observations of surface anatomy of in vitro sweetgum (*L. styraciflua*; Wetzstein and Sommer, 1982), red raspberry (*Rubus idaeus* L. 'Haida' x 'Canby'; Donnelly and Vidaver, 1984a), 'Silvan' blackberry (*Rubus* sp.; Donnelly *et al.*, 1986; Donnelly *et al.*, 1987), cherry (*P. cerasus*; Marin and Gella, 1988; Marin *et al.*, 1988), 'Queen Elizabeth' rose (*Rosa* sp.; Donnelly and Skelton, 1989), apple (*Malus pumila* Mill 'Bramley'; Blanke and Belcher, 1989), *Malus* sp. 'Ougnoe' (Kataeva and Butenko, 1987) and rose (*R. multiflora*) (Cappellades *et al.*, 1990) showed that stomates were spherical, widely open and raised above the surface of the epidermis as compared to more elliptical, sunken stomates of greenhouse or acclimatized plants. Stress lines were observed in the cuticle around the stomates in in vivo-grown leaves of 'Queen Elizabeth' rose (*Rosa sp.*) reflecting the activity of these stomates unlike in vitro-grown leaves (Donnelly and Skelton, 1989).

Microscopic examination of the stomates of *S. lacinatum* (Conner and Conner, 1984) and tobacco (*N. tabaccum*) (Pospisilova *et al.*, 1987) showed that stomates from greenhouse seedlings closed within 30 minutes and 4-8 minutes after cutting, respectively, while half of the stomates from in vitro *Solanum* remained widely open after 16 hours and took a long time to close in tobacco. Similarly, stomates of acclimatized cherry (*P. cerasus*) plants were closed at time zero after exposure to 45% RH, while only 75% of stomates from in vitro leaves closed after 15 minutes (Marin and Gella, 1988; Marin *et al.*, 1988). The rapid wilting of tissue-cultured plantlets immediately after transplanting was therefore attributed to the inability of their stomates to close rather than the reduction in wax components of leaf cuticles (Conner and Conner, 1984; Conner and Thomas, 1981).

As evidence was shown that EWs played a less important role than stomates in conserving water loss in IVPs, the relative contribution of stomatal versus cuticular component of transpiration was explored. These studies were necessary to find effective methods of acclimatization (Conner and Conner, 1984; Sutter, 1988). Pospisilova *et al.* (1987) associated acclimatization with reductions in stomatal transpiration which were larger than reductions in cuticular transpiration. Marin and Gella (1988), on the other hand, reported transpiration rates which were independent from stomatal closure.

By studying abaxial (stomatal) and adaxial (cuticular) conductances of in vitro apple 'Golden Delicious' (Sutter, 1988; Sutter *et al.*, 1988; Shackel *et al.*, 1990), cherry (*Prunus avium xpseudocerasus*), and sweetgum (Sutter, 1988; Sutter *et al.*, 1988) leaves, water loss appeared to occur mostly through the stomates of the three species immediately after removal from culture. Partial restoration of stomatal function was found upon acclimatization of the plants as detected by reduced stomatal conductances and microscopic observations, but the closure was incomplete and too slow to obviate water stress after transfer (Sutter, 1988; Sutter *et al.*, 1988).

Cuticular conductances were less and inconsistently affected by acclimatization than stomatal conductances (Sutter, 1988; Sutter *et al.*, 1988). Relative cuticular conductance formed 6% of stomatal conductance in sweetgum, 17% in apple and 55% in cherry (Sutter, 1988; Sutter *et al.*, 1988). The cuticular conductances of apple plantlets were within the normal range of field apple trees (Shackel *et al.*, 1990).

Slow stomatal closure was detected after removal from culture (Sutter, 1988; Sutter *et al.*, 1988; Marin and Gella, 1988; Shackel *et al.*, 1990). However, it was insufficient to control the large amounts of water loss by the plantlets and to avoid desiccation, especially at a more severe water stress of less than 90% RH. Water uptake, although normal, could not balance the excessive water loss occurring through the stomates.

Therefore, acclimatization of apple plantlets was done at 90% RH to avoid desiccation, since under normal conditions of transfer to low humidity, the rates of stomatal closure detected would be too slow to control water loss, desiccation and death (Sutter *et al.*, 1988; Shackel *et al.*, 1990). These observations showed that the major water loss occured due to lack of stomatal closure, and that successful acclimatization took place through restoration of stomatal function.

In in vivo plants, cuticular conductance was shown to be less important than stomatal conductance. It formed only 10% of stomatal fluxes and was ignored in studies of leaf resistance (Kolattukudy, 1970; Denna, 1970; Wenkert, 1983; Pearcy, 1983). An increase in cuticular resistance from 10 s cm^{-1} to 10000 s cm^{-1} would only increase the total resistance 10% with stomatal resistance of 1 s cm^{-1} (Hall and Kaufmann, 1975b).

b) <u>Reasons For Stomatal Malfunction In In Vitro Plantlets</u>

The reasons for stomatal malfunction in IVPs are unknown but most reports relate it to abnormal development of stomates due to the high humidity in the culture conditions (Wardle and Short, 1983). It has been suggested that the high humidity in culture affects the course of development of guard cells during leaf

maturation (Wardle and Short, 1983). This causes abnormal microfibril development of guard cell walls which develop according to a high-RH-open-stomate-state (Wardle and Short, 1983). Lack of guard cell wall elasticity has been shown since protoplasts responded normally to osmotic solutions, but not the cell walls (Ziv *et al.*, 1987). On the other hand, normal guard cell wall cellulose micrifibrils with radial arrangment similar to those in greenhouse and field guard cells were demonstrated in in vitro cherry (Marin and Gella, 1988; Marin *et al.*, 1988). In vitro guard cell walls, however, were thinner with less epicuticular wax than normal in vivo leaf types (Marin and Gella, 1988; Marin *et al.*, 1988). It is known that the guard cell wall cutinization and transpiration have a major role in the mechanism of stomatal opening and closing (Losch and Tenhunun, 1981; Cooke, 1983). Smith *et al.* (1990a; b) observed greater stomatal closure in acclimatized crysanthemum to be associated with thickening in the walls of the guard cells.

Ion imbalance in guard cells was also thought to cause stomatal malfunction (Wardle *et al.*, 1979; Wardle and Short, 1983). Guard cells of IVPs were found to accummulate starch (Marin and Gella, 1988), a phenomenon found in open stomates induced by high K^+ and Cl^- ion concentrations (Wardle and short, 1983; Pemadasa, 1981). Since tissue culture media contained high levels of ions and plant cultures were found to absorb large amounts of ions, then lack of stomatal closure was attributed to excess ions of K^+ and NH_4NO_3 (Wardle and Short, 1983) or $CaCl_2$ (Ziv *et al.*, 1987). An X-ray microprobe analysis of IVP guard cells showed a considerably increased ratio of sodium to potassium and calcium ions (Wardle *et*

al., 1979). Since K^+ and Ca^{+2} have important roles in the regulation of guard cell osmotic potential (Ψ_π) necessary for stomatal opening and closure, such ratios may prevent normal stomatal behavior.

Starch accumulation was found to be linearly related to stomatal aperture in many plants with swift changes in starch concentrations occurring in concert with stomatal movements (Losch and Tenhanen, 1981). The lack of starch breakdown into malate, as is normal in stomatal functioning, may be the cause of starch accumulation (Wardle and Short, 1981). However, malate has been reported to be formed by a separate pathway other than through starch breakdown (Jarvis and Morison, 1981).

Since ABA applications did not induce stomatal closure in IVPs, it was concluded that the stomatal closure mechanism was impaired such that endogenous ABA was ineffective in causing stomatal closure (Wardle *et al.*, 1979). However, endogenous amounts of ABA were not studied (Wardle *et al.*, 1979).

A possible influence of plant growth regulators on plantlet transplantability was also reported (Ziv, 1986; Conner and Thomas, 1981). Since cytokinins cause stomatal opening (Zeiger, 1983; Farquhar and Sharkey, 1982), it is possible that a residual effect of cytokinins from the multiplication stage may cause stomatal opening. However, definitive evidence has not been reported. There have been a few reports associating an increase in agar concentrations in the medium with improved acclimatization of IVP while a decrease in kinetin in the medium occurred without referring to stomatal function (Ziv *et al.*, 1983; Debergh *et al.*, 1981; Bornman and Vogelmann, 1984). Transferring plantlets to a hormone -free-

medium before transplanting into soil and its effect on improved survival may also be related to eliminating kinetin residues from plantlets (Hasegawa, 1980; Batlle and Aldrufeu, 1987; Maene and Debergh, 1987).

c) Control of Stomatal Responses

Stomates are the interface between the atmosphere and plants and have a major effect in plant interactions with their environment (Jarvis and Morison, 1981). Thus, stomates are the major perception sites to the various atmospheric conditions affecting the plant. The responses of stomates to atmospheric conditions is complex since the stomates have a peculiar function of controlling both CO_2 diffusion for Ps and water loss in plants (Farquhar and Sharkey, 1982; Salisbury and Ross, 1985; Pearcy, 1983). Stomates are influenced by factors which affect Ps; including CO_2 concentration, light and temperature, and factors which affect water loss; namely atmospheric water vapor and soil water stress or bulk leaf water potential (Ψ_w; Jarvis and Morison, 1981)

Stomates respond to light by opening, a mechanism which is biochemically linked to the photosynthetic process to allow for CO_2 conductance and Ps to take place (Jarvis and Morison,, 1981; Salisbury and Ross, 1985). Stomates have also been found to respond directly to light (Jarvis and Morison, 1981, Salisbury and Ross, 1985). Light may modify stomatal response to humidity through its effect on temperature (Losch and Tenhunun, 1981). High light intensities were found to reduce stomatal sensitivity to humidity (Losch and Tenhunun, 1981). Light may also affect stomatal closure indirectly through its longer term effects on leaf and cuticle structure. This influences peristomatal transpiration and thus guard cell

responses (Losch and Tenhunun, 1981). Thus, the low light intensity under which IVPs grow may influence the function of stomates through its influence on the cuticle structure of the guard cells and epidermis.

Stomates also adjust to open further at low CO_2 concentrations and close at high CO_2 concentrations. CO_2 concentration effects are modifiable by humidity where stomatal responses to low humidity interfere with CO_2 diffusion (Jarvis and Morison, 1981). Additionally, high concentrations of CO_2 were reported to be more effective on stomatal closure under low humidity than high humidity (Jarvis and Morison, 1981; Hall and Kaufmann, 1975a; Davies *et al.*, 1981).

Temperature also influences stomatal closure by modifying its responses to the various factors through its effect on the energy balance of the leaf, net Ps, and its indirect effect upon ambient humidity (Jarvis and Morison, 1981; Hall and Kauffman, 1975b). Temperature in in vitro culture may have minor influence on stomatal development and function of IVPs. This is because temperature is kept more or less constant (around 25° C) in the tissue culture environment. However, stomates were reported to be less sensitive to humidity at temperatures above 20° C (Hall and Kauffman, 1975b).

CO_2 concentrations were found to have little influence on stomatal responses to humidity (Jarvis and Morison, 1981). The low CO_2 concentrations detected during the light period in in vitro cultures may increase stomatal opening. Moreover, the near 100% RH of in vitro cultures may limit the response of the stomates to high CO_2 concentrations which occur at night and may offset the stomatal response to the dark (Hall and Kauffman, 1975a). Thus, factors like

humidity and water stress have the primary control over stomatal responses (Jarvis and Morison, 1981; Hall and Kaufmann, 1975a).

Stomates have a pivotal role in regulating water transport and water status in plants (Jarvis and Morison, 1981; Wenkert, 1983). The two stomatal responses which control leaf water status are: 1) stomatal response to changes in ambient humidity which affect the vapor pressure deficits (VPD) around the leaves and 2) water stress (Losch and Tenhunun, 1981).

Stomates are known to respond to VPD by direct perception of humidity mediated by epidermal leaf turgor which is thought to induce some metabolic change causing stomatal closure (Schulze *et al.*, 1987; Jarvis and Morison, 1981). Others reported that stomates respond to humidity by a hydropassive mechanism which is subsequently followed by changes in osmotically active solutes in the guard cells (Lange *et al.*, 1971; Wardle and Short, 1983; Losch and Schenk, 1978; Davies *et al.*, 1981). Thus, stomates were found to act as 'humidity sensors', the mechanism of which was reported to be through turgor relations of guard cells with epidermal cells. This mechanism depends on the pattern of water flow to and within the epidermis independently from the bulk water status of the plant (Losch and Tenhunun, 1981; Schulze *et al.*, 1987).

Regulation of stomatal opening was found to be through gradients in Ψ_w in the epidermis caused by transpirational water fluxes through the stomatal and peristomatal pore (Lange *et al.*, 1971; Schulze *et al.*, 1987; Losch and Tenhunun, 1981). Guard and subsidiary cells were found to have less cutinized cell walls which form major and differential water loss sites as compared to adjacent epidermal cells

(Losch and Tenhunun, 1981; Jarvis and Morison, 1981). An increase in evaporation demand changes water flux from that directed into the guard cells driven by their low vacuole Ψ_π to one which favors water loss through evaporation sites in the cell wall. A reduction in cell turgor and narrowing of stomatal pore thus results (Losch and Tenhunun, 1981). Thus transpirational flux occupies important role in stomatal regulation through its influence on Ψ_w gradients in the epidermis (Schulze *et al.*, 1987; Jarvis and Morison, 1981).

It is, therefore, expected that plants growing under high humidity with low transpirational fluxes, such as tissue culture plantlets, would have wide open stomates due to a consistent steady state turgor in the epidermal and guard cells, thus resulting in permenantly open stomates (Schulze *et al.*, 1987; Losch and Tenhanen, 1981). Moreover, since IVPs have been reported to have reduced epicuticular wax development (Sutter and Langhans, 1979, Grout and Aston, 1977) and stomatal guard cells with thin cell walls (Marin and Gella, 1988), the excessive epidermal and stomatal water loss in tissue culture plantlets must offset the epidermal turgor gradients necessary for stomatal closure. This shows the important role of the interaction between epicuticular wax and stomatal function and the need to restore both for proper acclimatization of IVPs. Under such conditions, an increase in cuticular wax in the epidermis and subsidiary cells, where water loss was reported to be higher than guard cells (Cooke, 1983), would be a valuable means for acclimatization of IVPs. Cutinization of the epidermal layer and guard cells were reported to have an important adaptive value in stomatal sensitivity to humidity (Losch and Tenhunun, 1981). Similarly, stomates of plants

living on wet sites were found to be least responsive to humidity (Losch and Tenhunun, 1981).

The direct response to humidity has been termed 'feedforward' regulation of stomatal closure and is known to reduce transpiration as evaporation demand increases thereby avoiding stress (Losch and Tenhunun, 1981; Jarvis and Morison, 1981, Lange *et al.*, 1971). Thus, induction of stomatal response to humidity in tissue culture plants is of extreme importance in reducing transpirational water loss. Stomatal response to humidity was found to have a great adaptive value in desert plants leading to water conservation and improved water use efficiency (Losch and Tenhunun, 1981; Losch and Schenk, 1978; Hall and Kaufmann, 1975a; b).

Stomates also respond to water stress by a 'feedback' regulation (Losch and Tenhunun, 1981). Transpiration is thought to cause reduced bulk leaf water content which results in a general turgor loss in the leaf affecting the guard cells of the plant. However, stomates were found to close only when very low (threshold) Ψ_w were obtained (Davies *et al.*, 1981; Jarvis and Morison, 1981). Under moderate transpiration, stomatal responses to humidity occurred independently from bulk leaf water status (Losch and Tenhunen, 1981; Schulze *et al.*, 1987). Stomatal responses to humidity occurred in isolated epidermal strips (Lange *et al.*, 1971). Moreover, stomatal closure occurred with increasing VPD whilst bulk Ψ_w remained constant (Jarvis and Morison, 1981; Losch and Tenhunun, 1981; Hall and Kauffman, 1975b). At very low Ψ_w, however, stomatal closure in response to water stress occurred independently from responses to humidity and stomates remained closed even in moist air (Losch and Tenhunen, 1981) and in the light (Davies *et al.*, 1981).

Stomates responded to water stress caused by reducing soil water status (Schulze *et al.*, 1987; Davies *et al.*, 1981). Although earlier reports showed that the response was due to a threshold Ψ_w, evidence is now showing that stomatal conductance is not mediated by mesophyll cells or xylem Ψ_w (Schulze *et al.*, 1987; Davies *et al.*, 1981). At critical water deficits, water stress was found to induce metabolic signals such as ABA or other chemical messenger causing stomatal closure, possibly by altering permeability of the membranes to ions (Davies *et al.*, 1981; Losch and Tenhunen, 1981; Schulze *et al.*, 1987). External applications of ABA to leaves resulted in closure of stomates (Davies *et al.*, 1981; Jones and Mansfield, 1970). When the Ψ_w of the leaves was maintained constant using mechanical pressure on roots with plants maintained in humid ambient conditions while their roots in water deficits stomatal responses to water deficits were still observed (Schultz *et al.*, 1987).

Under moderate water stress, feedback regulation was found to cause a reduction in a ceiling stomatal opening, and thus does not reduce transpiration in response to increased evaporation demand (VPD), but maintains a constant transpiration rate to prevent excessive water loss (Losch and Tenhunen, 1981). This type of response may be advantageous in inducing stomatal closure while hardening tissue culture plants in vitro. Indeed, inducing water stress in tissue cultured plants was used for in vitro hardening by reducing the Ψ_w of the medium. Ziv *et al.* (1983; 1987) and Wardle *et al.* (1983a) induced stomatal closure by reducing Ψ_w of the medium. However, since this mechanism establishes a ceiling stomatal opening at a reduced aperture and maintains a constant transpiration rate, it does not improve

stomatal functioning in terms of its response to VPDs. The value of this method in inducing normal functioning stomates in IVPs is questionable, yet it may be valuable in reducing water loss through maintaining a reduced rate of transpiration after transplanting.

3. Anatomical Features

a) Leaf Surface Anatomy

A variety of divergent anatomical features were observed in tissue culture plants which were associated in one way or another with the excessive water loss of the transplanted plantlets. The altered anatomy of in vitro plants was specifically related to the various environmental factors which prevailed during the course of morphogenesis of various plant structures. Among the anatomical features changed were stomatal size, stomatal frequency and distribution on leaf surfaces, epidermal surface cell configuration and the presence of surface structures such as trichomes and hairs. Differences in these features were shown in in vitro plants as compared to its in vivo counterparts and in most cases gradual changes to normal in vivo structures were observed as acclimatization proceeded.

In sweetgum (*L. styraciflua*) grown in vitro, stomates were circular with higher surface topography than the in vivo plants which had low-relief stomates (Wetzstein and Sommer, 1983; 1988). Stomates were larger in vitro and had significantly greater densities (number of stomates per mm^2) than in acclimated or field grown plants. These features would contribute to the water loss through the noted open nonfunctioning stomates (Wetzstein and Sommer, 1983; 1988). Brainerd *et al.* (1981), however, reported lower stomatal frequency (about 150

stomate per mm^2) in 'Pixie' plum (*P. institia*) plantlets than that of in vivo plants (300 stomates per mm^2). The larger size of stomates and the smaller size of epidermal cells observed in sweetgum may affect the stomates and hence stomatal counts (Conner and Conner, 1984).

Blanke and Belcher (1989) observed larger number of stomates in in vitro apple (*M. pumila*) than that of in vivo plants, but the frequency decreased drastically with leaf expansion. Thus the ratio of the number of stomates to that of the epidermis is more important (Manning *et al.*, 1977).

Stomatal index (stomatal number divided by number of epidermal cells and stomates) gave a better estimate of relative stomatal comparisons (Conner and Conner, 1984; Donnelly and Vidaver, 1984a). Larger stomates in in vitro-grown leaves but no differences in stomatal index were observed when in vitro and in vivo *S. laciniatum* were compared (Conner amd Conner, 1984). Similarly leaves of in vitro 'Silvan' blackberry (*Rubus* sp.) shoots during multiplication had half the number of stomates per leaf than that of in vitro plantlets. Greenhouse plants had 200 times the stomatal number of the rooted IVPs (Donnelly *et al.*, 1986). However, stomatal index was the same in all three treatments (Donnelly *et al.*, 1986).

In in vitro red raspberry (*R. idaeus*), the stomates were fixed open, slightly raised, amphistomatous, and occurred on the periphery of the leaf which would contribute to the water loss of transplanted plantlets (Donnelly and Vidaver, 1984a). In contrast, stomates of greenhouse seedlings predominantly occurred abaxially (hypostomatous) with few adaxial, and no pripheral stomates (Donnelly

and Vidaver, 1984a). The stomatal index was reduced in in vitro raspberry (5 to 50 adaxial, and 100 to 200 abaxial vs. over 350 abaxial in greenhouse plants). Upon acclimatizing in the greenhouse, new leaves had increased stomatal index and were hypostomatic while the persisting leaves did not change implying an irreversible in vitro anatomy (Donnelly and Vidaver, 1984a). Leaves which originated in culture and developed ex vitro had transitional surface features (Donnelly and Vidaver, 1984a). Fewer stomates were also observed in in vitro carnation (Leshem, 1983). Guard cells were raised in in vitro blackberry but flush with epidermal surfaces in in vivo-grown leaves (Donnelly *et al.*, 1986; 1987). Stomates were widely open in shoots on multiplication medium and more so on rooting medium compared to field plants (Donnelly *et al.*, 1986; 1987). In addition, fewer hydathodes with open pores and different internal anatomy which contributed to more water loss were found on the adaxial surfaces of in vitro blackberry, 'Totem' strawberry (*Fragaria xananassa* Duchesne; Donelly and Skelton, 1987) and 'Queen Elizabeth' rose (*Rosa* sp.; Donnelly and Skelton, 1989) when compared to in vivo plants of respective species.

Dhawan and Bhojwani (1987) observed a lower stomatal index of in vitro leaves of *L. leucocephala* in the multiplication medium which increased throughout rooting and subsequent acclimatization but remained lower than that of field grown plants even five months after acclimatization. Similarly, microscopic observations of leaf surfaces of rose (*R. multiflora*) showed that stomates of IVPs in stage II were spherical, open and raised above the surface (Capellades *et al.*, 1990). These features disappeared and approached the elliptical shape of the field plants with acclimatization treatments (Capellades *et al.*, 1990).

Hydathodes with open pores were found in IVPs which were not found on in vivo or acclimatized plants (Capellade *et al.*, 1990). SEM observations of abaxial leaf surfaces of in vitro apple *M. pumila* were found to have more than 95% deformed large circular stomates , raised 2 to 4 μm above the surface, with wide vestibules through which mesophyll cells showed and lacked ante-chambers (Blanke and Belcher, 1989). The observed stomates were distinctly larger than that of tree-grown leaves.

The abnormalities observed would evidently contribute to water loss of IVPs upon transplanting. This is also evidenced from the gradual change into normal anatomical features upon acclimatization (Donnelly and Vidaver, 1984a; Blanke and Belcher, 1990; Capellades *et al.*, 1990). Variations in stomatal size, stomatal frequency, or stomatal aperture were reported to account for differences in stomatal diffusion resistance (Siwecki and Kozlowski, 1973; Manning *et al.*, 1977; Volkenburgh and Davies, 1977; Farquhar and Sharkey, 1982). The significance of those findings also relate to their most likely association with the in vitro growth conditions. Understanding those conditions therefore, and how they quantitatively and qualitatively affect cuticular development, stomatal differentiation, and cellular anatomy in vitro would aid in finding methods to alleviate the abnormality and thus improve hardening of in vitro plants (Wetzstein and Sommer, 1983; Capellades *et al.*, 1990).

Stomatal development and frequency can be affected by environmental conditions such as water availability, light intensity, temperature, humidity (Amer and Williams, 1958; Penfound, 1931; Rea, 1921; Esau, 1977) and osmotic

concentration of the culture medium (Allsopp, 1954). Xeromorphic characteristics include higher stomatal frequency which respond to lowered humidity by closure (Esau, 1977).

Light intensity was reported to have pronounced effects on leaf and stomatal development (Lightenthaler *et al.*, 1981; Terry *et al.*, 1983; Knecht and O'leary, 1972). Stomatal index was shown to be determined by the quality and quantity of light incident on older leaves (Schoch, 1987). Leaves which developed under high light intensity have been reported to have greater stomatal numbers than leaves under low light levels (Esau, 1977; Fernandez and Mujica. 1973; Wild and Wolf, 1980; Wylie, 1951; Boardman; 1977). However, a study of light effect in vitro upon the surface structure of *Liquidambar* revealed that light was not the only factor affecting leaf development (Wetzstein and Sommer, 1983). Decreasing light intensity reduced the stomatal index of in vivo leaves drastically but resulted in slight changes to in vitro leaves (Wetzstein and Sommer, 1983). In vivo and in vitro leaves differed in stomatal index, starch accumulation and leaf thickness although they were at the same light intensity implying the effect of other factors in vitro which modify the light effect on in vitro plants (Wetzstein and Sommer, 1983).

Stomatal differentiation was reported to be affected by factors such as CO_2 concentrations, water relations, and hormone levels (O'leary and Knecht, 1981; Penfound, 1931). Humidity effects of the atmosphere and the medium on stomatal development have also been reported (Esau. 1977; Cutter, 1971). Leaves developing under low humidity and soil moisture had higher stomatal frequencies than those grown with ample moisture (Manning *et al.*, 1977; Banister, 1976;

Wangermann, 1961). Water potential of the medium has also been reported to affect leaf morphogenesis (Brown *et al.*, 1979) where normal stomatal development were induced by increasing agar concentrations of the medium (Leshem, 1983a; Ziv *et al.*, 1983). Light also was reported to affect the stomatal distribution where high light intensity caused hypostomatous leaves (Gay and Hurd, 1975). Esau (1977) reported the association between raised stomates and hydrophytic habitats; raised stomates were experimentally induced by enclosing developing leaves in vapor-saturated atmosphere.

Finally, the development of hydathodes and water pores is a humidity related phenomenon since hydathodes develop under high humidity as a method for water and mineral uptake when transpiration is suppressed (Donnelly and Skelton, 1989; Capellades *et al.*, 1990; Juniper, 1960).

One of the surface structural features observed to be modified in IVPs and which is normally related to water loss of plants is the development and distribution of hairs and trichomes (Donnelly and Vidaver, 1984a; Donnelly *et al.*, 1986). Donnelly and Vidaver (1984a) observed epidermal hairs, especially filliform types on abaxial surfaces of in vivo leaves of red raspberry which were almost completely absent in in vitro-grown leaves. Unicellular hairs were observed adaxially on leaf margins in greater numbers on field-grown plants than cultured plants. Glandular colleters were also more abundant on the abaxial surface of in vivo-grown leaves than on cultured leaves. Trichome number was greater in new leaves formed after transplantation and greatest in greenhouse- and field-grown plant leaves (Donnelly and Vidaver, 1984a). The presence of filliform and setose hairs would help reduce

transpiration upon transplanting by increasing the resistance of the boundary layer. Trichomes on the edges would also reduce hydathode water loss in the rosaceae species (Donnelly and Vidaver, 1984a). Tobacco plantlets grown in vitro lacked trichomes on their leaves as compared to seedlings (Pospisilova *et al.*, 1987).

Surface structure alterations have also been observed in leaf epidermis cell size and sinuation and undulation. Wetzstein and Sommer (1983) observed smaller irregularly shaped epidermal cells, with irregular, sinuous undulations in the anticlinal walls as compared to well-defined isodiametric epidermal cells in seedlings and in field-grown plants. Epidermal cells of in vitro red raspberry were polygonal or irregular in the main veinal areas with straight anticlinal walls and irregularly shaped in between the main veins with undulate anticlinal walls as compared to the more regularly shaped and sized epidermal cells of in vivo plants (Donnelly and Vidaver, 1984a). Abaxial epidermal cell outlines of greenhouse-grown 'Queen Elizabeth' rose leaves were rectilinear, while those of tissue-cultured plantlets were sinuous (Donnelly and Skelton, 1989). In addition, greenhouse-grown plant leaves had dark-staining substances scattered throughout the epidermial cells that were not present in cultured plantlet leaves (Donnelly and Skelton, 1989). Similarly, epidermal cells of *R. multiflora* varied in size and shape, but were smaller with few sinuous undulations in anticlinal walls while epidermal cells of in vivo and acclimatized plantlets were more regularly shaped, larger, longer and had more undulations (Capellades *et al.*, 1990).

The significance of the differences in epidermal structure observed in in vitro plants is unknown but is possibly related to epidermal cell size and leaf expansion.

The observed association between the epidermal structure in vitro and later acclimatization may indicate some significance of epidermal structure in plantlet hardening. Epidermal cell waviness has been attributed to mesophyll structure, to light, and to humidity (Esau, 1965). Watson (1942a) described light-shaded leaves with polyhedral walls which later displayed waviness during subsequent growth. This was attributed to differential hardening and unequal plasticity and cuticle expansion. Full-sun leaves hardened rapidly, fixing the polyhedral form of the walls forming regularly shaped cells and undulations (Wetzstein and Sommer, 1983).

b) Internal Leaf Anatomy

A modified leaf internal anatomy was one of the striking features observed in tissue culture plantlets. In vitro leaves were thinner, with no palisade layer or only one thin layer of poorly defined palisade cells as compared to the new growth of transplanted plants or greenhouse plants (Grout and Aston, 1978; Brainard *et al.*, 1981; Fabbri *et al.*, 1986; Donnelly and Vidaver, 1984a; Smith *et al.*, 1986; Dhawan and Bhojwani, 1987). Smaller undifferentiated mesophyll cells with extensive intercellular air spaces were also characteristic of in vitro leaves (Grout and Aston, 1978a; Brainerd *et al.*, 1981; Wetzstein and Sommer, 1982; 1988; Fabbri *et al.*, 1986; Donnelly and Vidaver, 1984a; Dhawan and Bhojwani, 1987). Cells from in vitro-grown leaves had large vacuoles, limited cytoplasmic content and had less ergastic substances than cells from seedling leaves (Wetzstein and Sommer, 1982). In vitro leaves of red raspberry *R. idaeus* were smaller, thinner and lacked supportive collenchyma tissues as compared with field and acclimated plants (Donnelly *et al.*,

1985). The latter had compact thick-walled colenchyma and sclerynchyma tissues in the veinal areas as well as the petioles and stems (Donnelly *et al.*, 1985).

Vitrified plantlets had a more extreme anatomy where leaves did not have palisade tissue, but only spongy, largely vacuolated mesophyll with large intercellular spaces (Gaspar *et al.*, 1987; Debergh *et al.*, 1981; Leshem, 1983a; Arnold and Eriksson, 1983). Vitrified plants had a succulent mantle-core type apex with large vacuolated cells (Leshem 1983a). Leaf primordia lacked procambium strands, and had few, abnormal vascular bundles (Leshem, 1983a). Deficiency of cellulose and hypolignification of the vessels and tracheids in vitrified plants were described. These were suggested to allow for more water uptake due to reduced cell wall pressure, causing hyperhydricity in vitrified plantlets (Kevers *et al.*, 1984).

Normal in vivo plants exhibited a thicker leaf structure (Pospisilova *et al.*, 1987) with a mesophyll well defined into palisade and spongy parenchyma layers (Wetzstein and Sommer, 1982; Fabbri *et al.*, 1986; Donnelly and Vidaver, 1984a). More dense, larger and compact mesophyll cells with few intercelluar spaces were shown. Additionally, abundance of tannin and phenolic substances in the cells was reported as compared to few or no ergastic substances in in vitro-grown leaves (Wetzstein and Sommer, 1982; 1983).

The abnormal anatomical characteristics seen in tissue cultured leaves correlate with the extensive water loss found in these plantlets (Wetzstein and Sommer, 1982; Brainerd *et al.*, 1981), and possibly with their observed poor photosynthetic rates (Grout and Aston, 1977; Wetzstein and Sommer, 1982; Smith *et al.*, 1986; Fabbri *et al.*, 1986). The large intercellular spaces which occurred in

IVPs had an extended cell wall surface which increased evaporation (Esau, 1977). It is known that lacunose cells decrease the diffusive resistance of gases, including water vapor, through the mesophyll cells (Dale, 1982). The large intercellular spaces and the reduced lignification may contribute to considerable deformation of leaves during desccation (Wetzstein and Sommer, 1983; Donnelly *et al.*, 1985; Capellades *et al.*, 1990). In addition, the reduced surface area of the mesophyll cells, the absence of palisade cells and the abnormal chloroplast structure, may reduce Ps (Dale, 1982; Wetzstein and Sommer, 1982). This in association with the raised and nonfunctional stomates and reduced cuticle may explain the abnormal behaviour of withering and death of in vitro-grown leaves upon transfer to ex vitro conditions (Wetzstein and Sommer, 1982).

Acclimatization is associated with the development of normal leaf structure which takes place only in the newly-formed leaves under ex vitro conditions (Wetzstein and Sommer, 1982; Fabbri *et al.*, 1986; Donnelly *et al.*, 1985). The abnormal anatomy of in vitro leaves is irreversible; persistent leaves and leaves which originated in culture maintained the in vitro leaf structure long after transplanting to the greenhouse (Grout and Aston, 1978a; Leshem, 1983b; Donnelly *et al.*, 1985; Fabbri *et al.*, 1986; Ziv *et al.*, 1983; Ziv, 1986). Successful acclimatization has been associated with the ability of the plants to produce new leaves (Grout and Aston, 1978a; Fabbri *et al.*, 1986; Desjardins *et al.*, 1988; Grout and Millam, 1985). This illustrates the importance of the environmental conditions in determining the proper ontogeny of IVPs competent in acclimatization.

The modified leaf anatomy of plants grown in vitro is typical for plants exposed to high humidity and low light in vitro (Conner and Conner, 1984; Smith *et al.*, 1986). Light intensity, humidity, moisture, temperature and nutrition have been shown to influence leaf anatomy (Sinnott, 1960; Dale, 1982; Schoch; 1987; Esau, 1977; Wangermann, 1961). In comparisons between shade and sun leaves, it was shown that leaves differentiated under low light intensity (shade leaves) were thinner than those differentiated at high levels, had less differentiated mesophyll, and a higher proportion of intercellular spaces (Dale, 1982; Jackson, 1967; McClendon and McMillen, 1982; Wylie, 1951; Bjorkman and Holmgren, 1963), similar to those found in IVPs. Low light levels were reported to decrease cell division in leaves resulting in reduced cell number. It also altered cell expansion from a plane perpendicular to one paradermal resulting in thinner leaves (Dengler, 1980; Dale, 1982).

High irradiance, on the other hand, increased cell number, size and leaf thickness due to differentiation of one or two layers of long palisade cells (Milthorpe and Newton, 1963; Dale, 1982; Terry *et al.*, 1983; Salisbury and Ross, 1985; Bjorkman and Holmmgren, 1963). In vitro plantlets exhibited typical shade leaf structure as compared to their normal colunterparts grown under natural conditions (Marin and Gella, 1988; Smith *et al.*, 1986).

Sweetgum (*L. styraciflua*; Lee *et al.*, 1988) cultures incubated at higher light intensities developed more normal palisade layers. Leaves of raspberry forming in the greenhouse under 9Klx developed palisade layer, but those grown under 3 and 6 Klx did not (Donnelly and Vidaver, 1984a). The differentiation of supportive

tissue was also influenced by light intensity with the amount of lignification being highest for the acclimatizing transplants grown in the higher light intensity (Donnelly *et al.*, 1985). In a study by Wetzstein and Sommer (1988) on the effect of light levels on the anatomy of in vitro plants, it was observed that both in vitro- and in vivo-grown leaves responded to low light levels by reducing mesophyll cell number, expansion and differentiation. However, in vitro leaves were thinner, had smaller cells, less palisade development, and generally lacked osmiophillic staining deposits as compared to in vivo leaves at the same light levels (Lee *et al.*, 1988). This indicated that factors unique to in vitro conditions may have affected the response of in vitro-grown leaves to low light levels. Similarly, seedlings growing under the same in vitro conditions as IVPs exhibited the typical in vitro anatomy of a reduced palisade layer (Grout and Aston, 1978a) or intermediate anatomy (Smith *et al.*, 1986) as compared to their field counterparts. This indicates environmental factors in addition to light affecting leaf anatomy (Smith *et al.*, 1986; Lee *et al.*, 1988; Grout and Aston, 1978a).

In addition to irradiance, it is reported that the number and differentiation of cells in a leaf are affected by CO_2 concentration, water relations, temperature, and nutrition (Esau, 1977; Dale, 1982; Schoch, 1987; Manning *et al.*, 1977). The number of cells in a leaf increases when the photosynthetic capacity of a plant increases through increased CO_2 concentration and light (Schoch, 1987).

Low soil moisture causes small number and size of cells through direct effect of water stress on cell division and expansion as well as on Ps and translocation (Dale, 1982; Manning et al., 1977). Xerophytic plants have thick leathery leaves

(Dale, 1982) with lignified tissues in the form of sclerenchyma associated with the vascular bundles and even the epidermal cells. In contrast, leaves growing in abundant moisture or high humidity lack such cellular thickenings (Esau, 1977; Donnelly *et al.*, 1985). Eberhardt (1903, cited by Esau, 1977) found reduced sclerenchyma fibers and secondary wall development including lignification in plants grown at high relative humidity. Wetzstein and Sommer (1983) Donnelly *et al.* (1985) and Capellades *et al.* (1990) observed epidermal cell collapse and distortion upon transplanting, which is related to lack of lignification of cells due to high RH.

Size of substomatal cavities has been correlated with humidity; enlarged area with high RH (Manning *et al.*, 1977). Changes in palisade mesophyll development of *Hedera helix* were also correlated with RH during leaf development (Watson, 1942b). Vitrified leaf characteristics were strongly related to water availability represented in Ψ_w of the medium or submergence of explants in water (Debergh *et al.*, 1981; Leshem, 1983a; Ziv *et al.*, 1983; Ziv, 1986).

Temperature was reported to affect leaf morphology of red raspberry in culture by inducing unifoliate instead of trifoliate leaves. This effect was not reversible upon transfer to ex vitro conditions, regardless of the light intensity (Donnelly *et al.*, 1985). However, temperature is kept around optimum for plant growth in tissue culture, although relatvely high, and has little effect on leaf morphology in vitro.

c) Root Anatomy and Vascular Connections

Desiccation of transplanted in vitro plants was suggested to occur as a result of water imbalance due to poor water uptake and excessive water loss (Wardle and

Short, 1983; Short *et al.*, 1987; Maene and Debergh, 1987). It is known that plant function depends fundamentally on plant water status, which in turn depends on the precarious balance between water loss to the atmosphere and water uptake from the soil (Soil-plant-atmosphere continuum; McKee, 1981; Wenkert, 1983; Davies *et al.*, 1981). In IVPs, poor control over water loss is due to several physiological and stuctural modifications induced by the in vitro environmental conditions. The role of roots and xylem tissues produced in vitro, in water uptake and transport, relative to the balance of water status of transplanted tissue cultured plantlets is therefore of crucial importance. Indeed, a return to a functional water balance after transplanting, and restablishment in soil will take place only if water absorption resumes through an effective and persisting root system, or after root replacement (Mckee, 1981; Pierik, 1988; McClelland *et al.*, 1990).

Good functional roots in in vitro plants were one of the determining factors for successful acclimatization of plantlets in soil (Walker *et al.*, 1987; Aldrufeu, 1987; Dunstan, 1981; Smith *et al.*, 1986; Roberts and Smith, 1990; Mohammed and Vidaver, 1990). It has been shown that plants with superior roots produced on cellulose rods (Sorbarod systems, Baumgartner papiers, CH 1001, Lausanne, Switzerland) and liquid medium lost more water, but wilted less than plants rooted on agar due to more efficient water uptake (Roberts and Smith, 1990). In vitro-grown roots were found to be modified, defective and nonfunctional (Aldrufeu, 1987; McClelland *et al.*, 1990; Donnelly *et al.*, 1985; Bowden, 1984; Maene and Debergh, 1983; Pierik, 1988), beyond the problems normally associated with transplanting (Flemer, 1982; Debergh and Maene, 1981; Roberts and Smith, 1990).

For successful establishment in soil, new roots had to be produced while plantlets underwent cessation of growth or desiccation (Debergh and Maene, 1981; Mckee, 1981). However, about 50% of roots of in vitro *Acer rubrum* L. 'Red Sunset' persisted after transplanting and allowed resumption of growth (McClelland *et al.*, 1990). Likewise, Donnelly (1985) reported persistent roots after transplanting, as well as newly produced roots which had transitional anatomy between in vitro and ex vitro roots. This indicates the importance of persistent roots in maintaining integrity of plantlets after transplanting, while new leaves and roots are being formed (McClelland *et al.*, 1990).

In vitro roots produced on agar solidified medium are known to lack root hairs (Aldrufeu, 1987; Barnes, 1979). Abnormal root morphology of loblolly pine (*Pinus taeda* L.) including lack of both lateral roots and a well-developed root system near the soil surface were reported to affect shoot growth and establishment in soil (McKeand and Wisnieski, 1981). In addition, loblolly pine had inefficient nitrogen and phosphorous uptake by in vitro-grown root systems (Mckeand and Allen, 1984). This was found to be due to reduced root surface area and not due to altered physiology. In vitro roots of three plant species studied by McClelland *et al.* (1990) produced roots with horizontal morphology which continued to distinguish these from ex vitro-grown roots during later stages of growth.

Grout and Aston, (1977) reported incomplete vascular development between roots and shoots of cultured cauliflower plantlets. This was possibly due to development of roots adventitiously from callus tissue rather than stem tissue as observed in some plant species (Hughes *et al.*, 1973; Aynsley and Marston, 1975).

When roots arose from callus, plantlets failed to survive upon transfer (Hughes *et al.*, 1973). Birchem *et al.* (1981) observed root-like formations in SEM studies of sweetgum which indicated morphologically different appendages lacking typical root appearance.

Roots of in vitro red raspberry were described to have a delicate appearance and covered with fine root hairs (Donnelly *et al.*, 1985). In addition, they were smaller in diameter, white or pinkish in color and had little periderm. In contrast, field plants were brown in color and had multilayered periderm. Roots of in vitro *Acer rubrum*, *Betula nigra* L., and *Malus xdomestica* Borkh 'McIntosh' were characterized by extremely enlarged cortical cells and consequently had a larger diameter than ex vitro roots (McClelland *et al.*, 1990). The vascular system of in vitro roots was underdeveloped as compared to ex vitro roots which produced vascular cambium and secondary growth (McClelland *et al.*, 1990). About one half of in vitro roots died after transplanting while the rest had collapsed cortex and new root extensions developed with ex vitro like structures. This shows the importance of functionability in in vitro-grown roots to avoid physiological imbalances during the transition period until new roots and leaves develop (McClelland *et al.*, 1990).

The vascular system of plantlet leaves and petioles was also shown to be poorly developed which affect water transport. Microcultured Asian white birch *Betula platyphylla* (Schneid.) Rehd. var. *szechuanica* had reduced leaf midribs containing xylem and phloem cells as well as small and poorly structured vascular connections in the petiole (Smith et al., 1986). This is in contrast to a larger more prominant vascular system in seedlings grown in the same in vitro conditions and

very well developed vascular connections in their greenhouse counterparts (Smith et al., 1986).

The poor vascular system development described by Smith *et al.* (1986) and McClelland *et al.* (1990) may contribute to in vitro plantlet susceptibility to water stress. Viable root systems are correlated significantly with acclimatization (Walker *et al.*, 1987; Aldrufeu, 1987; Hasegawa *et al.*, 1973; Mohammed and Vidaver, 1990; Smith *et al.*, 1990b). Roots also may restore the normal plant physiology since the roots can be a source of phytohormones such as kinetin and others induced by water stress (Marin and Gella, 1988; Ludlow *et al.*, 1989). The restoration of photosynthesis in microcultured Asian white birch plantlets, which occurred only after rooting, may be related to this last phenomenon (Smith *et al.*, 1986).

Root development and structure depend upon the physical characteristics of the rooting environment, as well as the plant species (McClelland, 1990). Whereas high light intensities were found to enhance root formation and acclimatization in *Sequoia sempervirens*, they inhibited root formation in several others (Walker *et al.*, 1987; Hasegawa *et al.*, 1973; Hansen *et al.*, 1978; Kataeva and Butennko, 1987). Sucrose was shown to be essential for root initiation and was found to enhance acclimatization of plantlets and increase survival (Aldrufeu, 1987; Desjardins *et al.*, 1987b; Rahman and Blake, 1988).

Because nutrients, water and oxygen are readily available in vitro, there may be no need to develop advanced secondary xylem and phloem to enhance conduction (McClelland *et al.*, 1990). The reduced transpiration in vitro due to the

high humidity was thought to cause ineffective water uptake in in vitro plants (Pospisilova *et al.*, 1987; Kozai, 1988). Several investigators tried to induce transpiration in vitro and thus normal water uptake by reducing the RH in the culture vessels or by increasing light intensity (Short *et al.*, 1987; Maene and Debergh, 1987). Lack of transpiration and subsequent lack of mineral uptake were reported to result in physiological disorders of plantlets in vitro (Kozai, 1988).

A histological study conducted by Marin and Gella (1988), of in vitro grown cherry (*P. cerasus*), showed normal vascular connections as well as effective water uptake. Similarly, Sutter (1988) and Shackel *et al.* (1990) reported normal water uptake. However, no work has been done to determine whether in vitro plantlets might have altered hydraulic resistances compared to greehouse or field-grown plants. Hydraulic resistance measures the change in Ψ_w of plant tissues with changes in transpiration rate (Passioura, 1980) and affects plant water balance. The lack of research in this area precludes any definitive conclusions concerning the water balances/imbalances which may occur in IVPs and their possible relation to root anatomy or physiology.

III. Heterotrophic Nutrition

a. Cell Cultures. Reports in the sixties and early seventies documented the heterotrophic growth of suspension cell and tissue cultures of higher plants (Venketeswaran, 1965; Fukami and Hildebrandt, 1967; Sunderland and Wells, 1968; Laetsch and Stetler 1965; Dalton, 1984). Heterotrophic tissues are defined as those that are dependent upon sugar as their only carbon source for nutrition and growth (Treat *et al.*, 1989). Photoautotrophic tissues are those which satisfy their carbon

requirement by photosynthetic fixation of CO_2 (Husemann, 1985; Treat *et al.*, 1989). Photomixotrphic tissues use sucrose as well as fix atmospheric CO_2 for their carbon source (Treat *et al.*, 1989). Autotrophic cultures indicates sucrose-free culture while heterotrophic cultures are sucrose-supplemented cultures. Photoautotrophic cell cultures were long sought for their importance in studying the physiology of Ps (Huseman, 1985; Horn *et al.*, 1983). In addition, photoautotrophic cultures have the potential for enhancing the synthesis of secondary metabolites (Fukami and Hildebrandt, 1967; Horn et al, 1983; Treat *et al.*, 1989).

Early attempts to induce heterotrophic cells and tissues to grow autotrophically by ommitting sugars from the culture medium were unsuccessful (Venketeswaran, 1965; Yamada and Sato, 1978; LaRosa *et al.* 1984). Sucrose was found to repress chlorophyll synthesis, chloroplast development and even directly photosynthetic capacity (Laetsch and Stetler, 1965; Fukami and Hildebrandt, 1967; Sunderland and Wells, 1968; Pamplin and Chapman, 1975; Yamada and Sato, 1978; Horn *et al.*, 1983; LaRosa *et al.*, 1984). In most cases, growth was totally dependent on sucrose and was reduced or ceased when sucrose was withheld from the medium even in the greenest tissue (Kaul and Sabharwal, 1971; Yamada and Sato, 1978; LaRosa *et al.*, 1984). Light quantity, sugar amount, CO_2 concentration and hormonal levels have been shown to affect photosynthetic activity of green tissues *in vitro* (Yamada and Sato, 1978; LaRosa *et al.*, 1984). Indeed, success in establishing autotrophic cell and callus cultures have been obtained only by the successive selection of green tissue (Yamada and Sato, 1978) and the absolute

requirement of elevated CO_2 levels and light intensity (LaRosa *et al.*, 1984; Horn *et al.*, 1983).

b. In Vitro Plantlets

Photoautotrophy in IVPs has received attention only recently (Solarova, 1989). Plantlets micropropagated in vitro grow heterotrophically on culture medium supplemented with sucrose. However, when transferred to the greenhouse they are expected to assume autotrophic nutrition (Grout and Aston, 1978b). Thus, these plantlets undergo a critical transition period after transfer ex vitro where they experience environmental stress with consequent low survival (Grout and Aston, 1977; 1978b; Brainerd and Fuchigami, 1981; Sutter and Langhans, 1979; Conner and Thomas, 1981; Wetzstein and Sommer, 1982).

The lack of autotrophic metabolism in IVPs has been suggested as a major factor, in addition to water stress, in the vulnerability of plantlets to environmental stress upon transplantation (Grout and Aston, 1978b; Wetzstein and Sommer, 1982; Marin and Gella, 1988). Evidence is the observation of poor photosynthetic rates in IVPs and their correlation with leaf degeneration after transplanting (Grout and Aston, 1978b; Grout and Millam, 1985; Marin and Gella, 1988). Furthermore, successful acclimatization of plantlets has been associated with photosynthetic activity in the persistent leaves (Grout and Circus, 1988) and/or the speed of development of photoautotrophic new leaves (Grout and Circus, 1988; Grout and price, 1987; Donnelly and Vidaver, 1984b).

Low or no photosynthetic rates (net negative carbon balance) were reported in in vitro cauliflower (Grout and Aston, 1978b), red raspberry (*R. idaeus*; Donnelly

and Vidaver, 1984b, Donnelly *et al.*, 1984), strawberry (Grout and Millam, 1985) and African violet plantlets (Khokhar, 1983). In these plantlets, in vitro-formed leaves failed to assume autotrophic nutrition or support growth after transplanting. They became yellow, necrotic and degenerated, leading to death of the plantlets (Grout and Aston, 1978b; Grout and Millam, 1985; Grout and Donkin, 1987). Similarly, IVPs of brassicas, african violet, strawberry (Grout and Millam, 1985; Grout and Price, 1987) and *Rosa* hybrid (Langford and Wainwright, 1987) failed to grow autotrophically and died within four to eight weeks after transfer to sucrose-free medium. In vitro cherry (*P. cerasus*), plantlets died 15 days after transplanting even though the critical water stress period had passed (Marin and Gella, 1988). Persistent leaves of in vitro red raspberry (*R. idaeus*) remained net respirers after one month from transplanting (Donnelly and Vidaver, 1984b). This evidence indicates the importance of photosynthetic competence on survival of IVPs.

Only plantlets such as *Dieffenbachia picta*, potato (*Solanum tuberosum*) and *Chrysanthemum*, whose in vitro-grown leaves demonstrated a positive carbon balance were able to persist after transplanting and support survival (Grout and Circus, 1988). Survival of strawberry, cauliflower and african violet plantlets however, depended upon the development of new leaves, under post-transplant conditions, which were capable of fixing CO_2 (Grout and Circus, 1988). Reduced photosynthetic rates of in vitro-derived red raspberry (*R. idaeus*) leaves were found to persist through acclimatization while photoautotrophy developed gradually in the new successive leaves formed ex vitro (Donnelly and Vidaver, 1984b, Donnelly *et al.*, 1984). In vitro-grown leaves were considered to be of little survival value and

to be transitory pseudo-cotyledonary leaves, which acted as a nutrient source for the development of new leaves (Wardle *et al.*, 1979; Wardle *et al.*, 1983b; Grout and Millam, 1985).

Acclimatization of *L. leucocephala* was marked by the accumulation of starch granules which was considered an indication of photosynthetic activity (Dhawan and Bhojwani, 1987). Enhancing Ps in vitro was reported to increase dry matter accumulation in the roots, stems and leaves which was suggested to help the plant survive when transplanted to soil in the greenhouse (Mousseau, 1986; Kozai *et al.*, 1988a; Tolley *et al.*, 1984a; b).

In contrast, Lee *et al.* (1985) reported that lack of photosynthetic capacity was not a limiting factor in plantlet acclimatization and transplant growth of *L. styraciflua* since plantlets exhibited higher rates of Ps than seedlings grown under the same light intensity. Evers (1982) reported that in vitro maximum net Ps of Douglas fir plantlets reached the same level as reported for trees. Ps was not modified by the in vitro environment. Grout and Circus (1988) distinguished between two groups of plants; those whose Ps system is active in vitro and adapt easily to ex vitro conditions, and those whose leaves formed in vitro never adapt to Ps activity when transplanted.

Direct association between photoautotrophy and acclimatization, however, has not been proved. Fujiwara *et al.* (1988), although succeeding in inducing complete autotrophy in plantlets growing in vitro, failed to detect reduction in water loss in detached leaves in response to water stress. Kozai *et al.*, (1987a) and Hayashi and Kozai (1987) reported improved growth of strawberry and taro

plantlets growing autotrophically in a preparation stage after transplanting, but failed to present sufficient experimental results validating the effect of autotrophy on acclimatization. Desjardins *et al.* (1988) failed to detect acclimatization features in in vitro autotrophic strawberry and raspberry although increased stomatal density was observed.

Reasons for low photosynthetic rates in IVPs appear to be diverse. Cauliflower plantlets had poor development of the photosynthetic system with reduced chlorophyll content, limited reduction of 2,6-dichlorophenolindophenol (DCPIP) in the Hill reaction, and a net loss of CO_2 into the air (Grout an Aston, 1978b). Both cauliflower (Grout and Aston, 1978b) and raspberry (Donnelly and Vidaver, 1984b) plantlets develop net CO_2 uptake, but only in new leaves after transplanting. This implies an irreversible impairment of Ps machinery in the in vitro-formed leaves.

Abnormalities in chloroplast structure and palisade layer have been reported. *L. styraciflua* plantlets were observed by electron microscopic to have flattened chloroplasts with an irregularly arranged internal membrane system when compared to acclimatized or field-grown leaves (Wetzstein and Sommer, 1982). Irregular development of the palisade layer has been reported in *B. oleracea* (Grout and Aston, 1978a), Plum (*P. institia*; Brainerd *et al.*, 1981) and *L. styraciflua* (Wetzstein and Sommer, 1982). The abnormal internal chloroplast membrane development and minimal differentiation of the palisade parenchyma suggested that the photosynthetic capacity of these plantlets may be lower than that of in vivo plants (Wetztein and Sommer, 1982). Smith *et al.*, (1986) observed reduced photosynthetic

rates in in vitro shoots of white birch (*B. platyphylla*) on a fresh weight basis as well as on a chlorophyll basis when compared to seedlings. This indicated additional differences, other than those due to palisade differentiation (probably involving chloroplast functioning) were also involved (Smith *et al.*, 1986).

Contrary to the previous studies, Lee *et al.* (1985) showed that cultured plants had functional chloroplasts with photosynthetic capacity higher than that of seedlings. Grout and Price (1987) and Grout and Donkin (1987) reported normal levels of light-stimulated electron transport in in vitro cauliflower and strawberry which were comparable to those of seedlings. This indicated normal thylakoid structure and function. However, reduced levels of chlorophyll and ribulose bisphospate carboxylase-oxygenase (Rubisco) activity resulted in low carbon assimilation (Grout and Price, 1987). New leaves that formed on sucrose-free medium developed a positive carbon balance and increased Rubisco activity and chlorophyll content (Grout and Price, 1987).

In other studies, however, chlorophyll content could not be regarded as a limiting factor in Ps. Uptake of CO_2 of in vitro *Rosa* sp. increased with reduction of sucrose in the medium while chlorophyll concentration decreased, possibly due to improved chlorophyll efficiency (Langford and Wainwright, 1987). The improvement in CO2 uptake could not be related to chlorophyll alone (Langford and Wainwright, 1987). Microcultured shoots of Asian White Birch increased their Ps dramatically after transplanting and rooting while chlorophyll concentration remained the same (Smith *et al.*, 1986). Conner and Thomas (1981) indicated that "even if chlorophyll is present in the leaves, it is probable that the enzymes

responsible for photosynthesis are inactive or absent". Similarly, Bjorkman and Holmgren (1963) found no correlation between chlorophyll concentration and photosynthetic capacity and Husemann (1985) showed that high chlorophyll content was not always a reliable index of photosynthetic competence of cell cultures.

Later studies indicated that the low photosynthetic rates obtained in IVPs were not due to impairment of the photosynthetic machinery (Pospisilova *et al.*, 1987; 1988; Smith *et al.*, 1986; Fujiwara *et al.*, 1987). Full development occurred, but the low Ps in vitro was associated with the limitations in the environmental conditions of in vitro cultures (Pospisilova *et al.*, 1987; 1988; Smith *et al.*, 1986; Fujiwara *et al.*, 1987). Smith *et al.* (1986) observed complete recovery of Ps in in vitro birch after transfer to the non-limiting gaseous conditions ex vitro. Similarly, Pospislova *et al.* (1987), working under non-limiting CO2 and light conditions, found comparable Ps rates between tobacco plantlets grown on sucrose-free medium and tobacco seedlings. However, the lower biomass of in vitro tobacco indicated that low Ps rates occurred in vitro, possibly due to the effect of environmental conditions in microculture rather than due to low photosynthetic capacity (Pospisilova *et al.*, 1987). Likewise, although higher Ps rates were observed for *L. styraciflua* plantlets than seedlings when measured under saturating conditions, plantlets in culture displayed comparably lower vigor than seedlings (Lee *et al.*, 1985). This reflected the low Ps activity associated with restricted in vitro conditions (Lee *et al.*, 1985).

A carbon dioxide response curve of in vitro tobacco revealed lower CO_2 saturation point and higher compensation point than seedlings. This behavior was similar to that of shade plants, and was associated with the low light levels of in

vitro conditions (Posilisova *et al.* 1987; 1988). In a similar study, Ps in in vitro tobacco and potato plantlets were significantly lower, CO_2 compensation points greater, and Ps saturation points lower than those of acclimated transplants (Pospisilova *et al.*, 1988). However, fluorescence studies of chlorophyll a revealed that the Ps apparati in both in vitro tobacco and potato plantlets were fully developed. Large increases in Ps occurred after transplanting and the photosynthetic parameters approached those of seedlings (Pospisilova *et al.*, 1988). Dhawan and Bhojwani (1987) observed photosynthetic activity, as indicated by accummulation of starch in mesophyll cells, only after plants were transferred outside the culture vessels to be acclimatized. Thus, these results showed that the low Ps detected in early studies was due to environmental conditions in vitro which restricted the Ps activity.

Lettuce seedlings grown on agar-medium in culture vessels had lower maximum Ps rates and photosynthetic efficiency than vermiculite-grown seedlings, thus indicating the influence of in vitro environmental conditions (Aoki and Oda, 1988). Seedlings of birch grown openly in a growth chamber under the same light and humidity conditions as in vitro cultures, had photosynthetic rates similar to greenhouse plants (Smith *et al.*, 1986). This would indicate that the in vitro gaseous atmosphere affects the Ps of micro-cultured plants.

The above reports support the conclusion that IVPs have fully developed Ps system and that the low Ps rates are due to the influence of specific in vitro environmental conditions on the Ps process (Kozai, 1991a; b).

c. Effect of Environmental Conditions on Photoautotrophy of IVPs

In vitro environmental conditions which have been suggested to limit photosynthetic rates and possibly Ps capacity of IVPs include the following: organic carbon source, low light intensity, restricted gas exchange, oxygen concentration, nutrient salt medium, temperature and exogenous hormones (Grout and Aston, 1978b; Donnelly and Vidaver, 1984b; Lee *et al.*, 1985; Grout and Donkin, 1987; Kozai, 1991a). These same conditions have been reported to affect Ps competence of cell cultures (Husemann, 1985; Yamada and Sato, 1978).

The presence of sucrose as an exogenous carbon source in tissue culture media is thought to be the major factor involved in the heterotrophic/photoautotrophic development of IVPs (Grout and Price, 1987; Grout and Donkin, 1987). Plantlets in vitro depend completely (heterotrophic) or partly (mixotrophic) on sucrose for their growth (Langford and Wainwright, 1987; Kozai and Iwanami, 1988; Mousseau, 1986). Numerous reports have demonstrated the reduced growth of plantlets or lack of survival upon ommission of sucrose from growth medium or transfer to soil (Grout and Millam, 1985; Grout and Price, 1987; Langford and Wainwright, 1987). Evers (1982) showed that sucrose levels affected rates of Ps of Douglas fir with lower concentrations of sucrose correlated with higher potential photosynthesis. Langford and Wainwright (1987) failed to induce photoautotrophic growth in tissue cultures of roses by eliminating sucrose completely, but obtained increased Ps by reduction of sucrose from 40 to 10 g/L. Leaves of strawberry, cauliflower, and African violet formed in vitro from sucrose-containing medium did not survive upon transfer to sucrose-free medium (Grout

and Price, 1987). However, new leaves developing on sucrose-free medium from defoliated plantlets achieved positive carbon balance and autotrophic growth (Grout and Price, 1987). Even these new leaves did not grow autotrophically when returned in vitro on sucrose-containing medium (Grout and Price, 1987) This indicated the inhibitory effect of sucrose on photosynthetic activity of plantlets.

In vitro tobacco plantlets cultured on sucrose-free media had small differences in Ps rates and Ps parameters compared to seedlings. In contrast, potato plantlets grown on sucrose-supplemented media differed dramatically in photosynthetic rate and parameters from potato seedling grown ex vitro, indicating the effect of sucrose in reducing photosynthetic competence of IVPs (Pospisilova *et al.*, 1988). In another study, seedlings of cauliflower grown in culture vessels in vermiculite (sucrose-free) had normal photosythetic rates as compared to plantlets grown on sucrose (Grout and Aston, 1978b). This indicated that the problems with photosynthetic activity in in vitro plantlets were directly related to leaf development in the presence of exogenous sucrose (Grout and Millam, 1985; Grout and Donkin, 1987); if they developed under sucrose-free medium they developed normal Ps.

Grout and Price (1987) and Grout and Circus (1988) have suggested that the sucrose effect resulted from the suppression of Rubisco activity or synthesis, and to a lesser extent, phosphoenol pyruvate carboxylase (PEPcase; Grout and Circus, 1988; Neumann and Bender, 1987). In cell cultures, sucrose was found to inhibit chlorophyll synthesis and/or directly the photosynthetic process (LaRosa *et al.*, 1984; Husemann, 1985). In plantlet cultures, however, chlorophyll content seemed

to increase with increasing sucrose (Langford and Wainwright, 1987; Mousseau, 1986).

A minimum concentration of sucrose, however, was reported to be necessary for the development of autotrophic metabolism in in vitro plants (Langford and Wainwright, 1987; Solarova, 1989; Kozai *et al.*, 1988b). It is possible that, in some plants, sucrose is needed initially in the medium for supporting growth and morphogenesis of shoots and leaves necessary for Ps (Solarova, 1989). Langford and Wainright (1987) found that plantlets pre-grown at higher sucrose (20 and 40 g l^{-1}) concentrations adapted better to autotrophic exposure and gave higher CO_2 uptake than those pre-grown on lower sucrose (10 g l^{-1}). This occurred even though the latter had better CO_2 uptake in vitro. The adverse effect of low sucrose (10 g l^{-1}) on growth of plantlets in vitro inhibited Ps when transferred to autotrophic medium (Langford and Wainwright, 1987). In vitro carnation (*D. caryophyllus*; Kozai *et al.*, 1988b; Kozai and Iwanami, 1988) and *Cymbidium* (Kozai *et al.*, 1990b) grown under non-limiting conditions of light and CO_2 had improved growth compared to plantlets grown at the limiting in vitro conditions. This improvement occurred at all sucrose concentrations, with highest growth at 1% sucrose and to a lesser degree at 0%, with 2% inducing the lowest growth. This indicated the relative superiority of mixotrophic and autotrophic nutrition over heterotrophic (Kozai and Iwanami, 1988; Kozai *et al.*, 1988b).

On the other hand, tobacco plantlets were shown to have well developed Ps apparatus, regardless of sucrose concentrations during growth period. However, their growth was reduced with decreasing sucrose concentration (Solarova, 1989).

This indicated that other conditions for autotrophic growth become more limiting to growth at low sucrose concentrations (Solarova, 1989; Kozai, 1988). The more limiting the in vitro environmental conditions such as CO_2 and light intensity, the more dependent growth becomes upon sucrose (Mousseau, 1986; Kozai, 1988; Kozai *et al.*, 1987b; c; 1988a; Solarova, 1989).

Light intensity is another important environmental factor influencing development of photosynthetic activity in IVPs. It is well known that photosynthetic characteristics of plants are influenced by the light climate in which they are grown (Salisbury and Ross, 1985; Boardman, 1977). The rate of Ps, palisade and mesophyll anatomy, chloroplast ultrastructure, chlorophyll content, Rubisco activity, and possibly stomatal diffusion to CO_2 are all influenced by light intensity (Bjorkman and Holmgren, 1963). Different light response curves were observed for sun and shade leaves although the slopes (efficiency of the photochemical process) were the same (Bjorkman and Holmgren, 1963; Salisbury and Ross, 1985). High light intensity was found to cause photoxidation and specific damage to light harvesting pigments (bleaching of chlorophyll) in shade leaves which lead to leaf death (Salisbury and Ross, 1985).

The low light intensity of tissue culture environment was found to confer shade-plant-like characteristics to the plantlets (Smith *et al.*, 1986; Pospisilova *et al.*, 1987; 1988). This included lower light saturation (maximum net Ps) and compensation points (Smith *et al.*, 1986; Pospisilova *et al.*, 1987). This implied that increasing light intensity in vitro or ex vitro would enhance Ps and possibly

acclimatization (Donnelly and Vidaver, 1984b; Donnelly *et al.*, 1984; Desjardins *et al.*, 1987a ; 1988; Kozai and Iwanami, 1988).

However, attempts to improve Ps rates in vitro or ex vitro by increasing light intensity have resulted in complete or partial failures (Donnelly and Vidaver, 1984b; Donnelly *et al.*, 1984; Desjardins *et al.*, 1987a; Lakso *et al.*, 1986; Kozai and Sekimoto, 1988). Donnelly and Vidaver (1984b) could not increase rates of Ps of IVPs by increasing light intensity in vitro, nor could they induce in vitro-derived leaves to respond to light increases (Donnelly *et al.*, 1984). Nevertheless, newly formed leaves, after transplanting IVPs as well as leaves from field-grown plants responded to increased light intensity by increasing CO2 uptake, dry matter accumulation, and pigment content (Donnelly and Vidaver, 1984b; Donnelly *et al.*, 1984). This indicated the importance of the environmental conditions (including other factors than light) under which in vitro leaves develop, relative to Ps activity of plantlets. Lakso *et al.* (1986) and Desjardins *et al.* (1987a) induced a delayed promotion in Ps in in vitro transplants by increasing photosynthetic photon flux density (PPFD) levels in the greenhouse, but this was combined with CO_2 enrichment as well.

Lee *et al.* (1985) observed higher Ps rates and altered anatomy in IVPs of *L. styraciflua* compared to seedlings grown under the same light intensity. This indicated that light intensity was not the only in vitro factor affecting the development of plantlet capacity for autotrophic metabolism. When all other conditions were changed upon transfer ex vitro, new leaves developed

photosynthetic activities which responded normally to light (Donnelly and Vidaver, 1984b, Donnelly *et al.*, 1984).

The above evidence indicated that either sucrose ommission or light augmentation alone fail to enhance Ps. The physical factors affecting growth and development of plant cultures are not independent and interact in a complex way (Hughes, 1981). Detailed ecophysiological studies, therefore, were carried out to elucidate the physical characteristics of the culture environment. The CO_2 concentrations in particular as well as the influence of a combination of factors (light, sucrose, CO_2, salt medium, oxygen, temperature and humidity) on plantlet Ps and acclimatization in vitro (Fujiwara *et al.*, 1987; Kozai *et al.*, 1986; Pospisilova *et al.*, 1987; 1988; Desjardins *et al.*, 1988; Solarova, 1989; Kozai *et al.*, 1990b) were studied.

The limited permeability of tissue culture vessels to CO_2 has been reported to be an important factor contributing to the abnormal physiology and consequent low survival of IVPs (Grout and Aston, 1978b; Abbott and Belcher, 1981; Donnelly and Vidaver, 1984a; Lee *et al.* 1985; Kozai *et al.*, 1986; 1987; Lakso *et al.*, 1986; Mousseau, 1986; Fujiwara *et al.*, 1987; Desjardins *et al.*, 1988; Pospisilova *et al.* 1987; Solarova, 1989; Walker *et al.*, 1988). Abbott and Belcher (1981) reported marked CO_2 fluctuation in the closed system of tissue cultures. A drop from 10,000 ppm to 500 ppm in the first hour of light was observed. This suggested that plantlets in culture spent most of the light period at CO_2 concentrations near photosynthetic compensation point where no net Ps took place (Abbott and Belcher, 1981).

Fujiwara *et al.* (1987) studied the CO_2 levels in closed micropropagation vessels during the culture of eight genera of ornamental foliage. They found that the CO_2 level increased to about 3000 to 8000 ppm in the dark period and was reduced to levels as low as the CO_2 compensation point (ca 30-80 ppm) in one to two hours in the light. Desjardins *et al.* (1988) also measured low CO_2 concentrations of 100 ppm after two hours of light. Solarova (1989) reported CO_2 concentrations to drop from about 624 ppm to about 149ppm in the first three to four hours of light. This resulted in the plantlets remaining in a state of starvation for CO_2 through 60 to 70% of the light period thus retarding their autotrophic growth. Concentration of CO_2 decreased to about 100-200 ppm for strawberry plantlets (Kozai and Sekimoto, 1988) even when cultures were capped with relatvely gas permeable caps. Similar results have been obtained for statice (*Limonium* Hybrid; Kozai et al, 1987b), *Cymbidium*; Kozai *et al.*, 1987c), and carnation (Kozai and Iwanami, 1988) where CO_2 concentrations of 100 to 150 ppm were measured during the light period.

The fact that CO_2 levels decreased after the onset of the light period showed that most plants had photosynthetic ability in vitro, but could not acheive their full Ps capacity due to limiting CO_2 concentrations in the tightly closed vessels (Fujiwara *et al.*, 1987). The low CO_2 concentrations limited the response of plantlets to increased light intensity. Under those conditions, sucrose was indispensible for growth of plantlets in vitro thus the failure of plantlets to assume autotrophy upon ommision of sucrose.

At low CO_2 concentrations, near the CO_2 compensation point, no net Ps takes place, and increasing PPFD is ineffective in causing any net CO_2 fixation (Salisbury and Ross, 1985; Kozai, 1989; Kozai *et al.*, 1990a). Rubisco activity is also known to be low at low CO_2 concentrations (Farquhar and Sharkey, 1982). An interacting relationship exists between CO_2 concentrations and light intensity; Ps is more responsive to light at higher CO_2 concentrations than at low CO_2 concentrations (Salisbury and Ross, 1985). Further, high CO2 concentrations causes greater maximum net Ps at higher light intensities (Salisbury and Ross, 1985). Net photosynthetic rates, dark respiration rates and sugar uptake are also interrelated and influenced by physiological (leaf area, dry weights, etc.) and environmental (light, CO_2, humidity, etc.) factors (Kozai *et al.*, 1988b). Dunwell (1979) observed lower productivity in in vitro tobacco seedlings when he removed CO_2 from the culture vessel atmosphere.

As noted earlier, studies by Posilsova *et al.* (1987; 1988) and Solarova (1989) reported full development of the Ps systems despite the high sucrose and low light intensity levels. Low CO_2 concentrations in the culture vessel formed the major limitation for autotrophic growth in the absence of sucrose (Fujiwara *et al.*, 1987). Inferences about CO_2 limitation in the culture vessels were also made by the several studies carried out by Kozai and co-workers and others (Mousseau, 1986) where significant increases in Ps and growth of plantlets were obtained by enrichment of the in vitro culture environment with CO_2 (Kozai *et al.*, 1990a; Kozai *et al.*, 1987b; Kozai *et al.*, 1987c; Kozai *et al.*, 1988b; Kozai and Iwanami, 1988; Kozai, 1988). Under conditions of CO2 enrichment and sucrose elimination, plantlets responded

better to increased PPFD by increasing their Ps, and did not need sucrose for their growth, i.e. they developed autotrophy (Kozai *et al.*, 1988a; Fujiwara *et al.*, 1988; Honjo *et al.*, 1988; Kozai, 1991b). According to Kozai and Iwanami (1988), sucrose was indispenable for the plantlet growth only when CO_2 and light were not sufficient for autotrophic growth.

Oxygen concentration in the vessel was also found to influence the autotrophy. Lowering the oxygen concentration in the in vitro vessel from 21% to 1% increased fresh weight, net photosynthetic rate, dry weights and leaf area per plantlet since it decreased photorespiration in C_3 plants (Shimada *et al.*, 1988). Photorespiration, resulting from competition of O_2 with CO_2 for Rubisco, is known to reduce the the photosynthetic activity in C3 plants especially under reduced CO2 concentrations (Salisbury and Ross, 1985; Shimada *et al.*, 1988). This situation was also observed in in vitro cultures (Evers, 1982; Pospisilova *et al.*, 1987; 1988).

The salt nutrient solution used in microcultures has also been shown to interact with the in vitro environmental conditions. A Comparison of full or half strength Murashige and Skoog nutrient solution (MS; Murashige and Skoog, 1962) with Enshi hydroponic solution (Hori, 1966), under nonlimiting autotrophic conditions, resulted in increased Ps, fresh weights, root formation, and decreased dark respiration of carnation plantlets (Kozai *et al.*, 1988b). Salt mixtures formulated for autotrophic hydroponic cultures, therefore, were more suitable than MS basal salt mixture, which was developed for supporting the heterotrophic growth of in vitro cultures.

The gaseous environment in micropropagation vessels is limited by tightly fitting lids and often with plastic film wrap which further reduces gas infiltration (Walker *et al.*, 1988). Gas infiltration is thus only driven by the expansion and contraction of gases within the vessel as influenced by ambient temperature which is generally limited due to the narrow range in the culture room (Walker *et al.*, 1988). The vessel-stopper combination has been found to affect the gas environment of plantlets in vitro and thus plantlet growth and development. It alters the gas exchange between the air of the room and the air in the vessel (Kozai *et al.*, 1986). Kozai *et al.* (1986) measured gas exchange by the rate of air infiltration (number of air changes; Ec) and the coefficient of gas exchange (Qc) using CO_2 as a tracer. The number of air changes was found to be dependent upon gas concentration outside the vessel, and was greater for smaller vessels and the most permeable stoppers (plastic formed cap as compared to aluminum foil, silicon foam or rubber plug). Coefficient of gas exchange of various vessel-stopper combinations was found to vary considerably with the stoppers, but not the vessel size. Vessels covered with plastic cap had a Qc of 10-fold that covered with aluminum cap (Kozai *et al.*, 1986). The gas exchange in combinations of six vessels with three lids resulted in gas infiltration ranging from 0.042 to 1.5 gas exchanges per hour (Kozai *et al.*, 1986). Experiments involving in vitro CO_2 enrichment necessitate the use of gas permeable closures to ensure increased CO2 concentration in the culture vessel (Kozai and Sekimoto, 1988; Kozai *et al.*, 1988a; b, Kozai, 1988; Kozai *et al.*, 1990a; b).

Temperature is known to promote Ps enzymes kinetically for both C3 and C4 plants (Salisbury and Ross, 1985). However, Ps enhancement is offset by the increase in the solubility of O_2 to CO_2 ratio at higher temperatures, which increases photorespiration in C3 plants (Salisbury and Ross, 1985). High temperatures also adversely affect electron transport and photophosphorylation, while low temperatures reduce the activity of Rubisco enzyme (Farquhar and Sharkey, 1982). Photosynthetic rates of *Cymbidium* plantlets decreased at temperatures of 15 °C and 35 °C at all light levels, but increased at an optimum of 25 °C. At 35 °C, the CO_2 compensation point was increased two-fold of that at 25 °C, and net Ps rate was close to zero at ambient CO_2 concentrations regardless of light levels (Kozai *et al.*, 1990b). Decreasing temperature at night (15 °C) was suggested to decrease the dark respiration and thus enhancing the net positive CO_2 balance (Kozai, 1991b).

High relative humidity of culture vessels were reported to affect Ps through its influence on reducing transpiration rates and consequent nutrient uptake necessary for Ps (Pospisilova *et al.*, 1987; Kozai, 1988; Kozai, 1991a; Kozai, 1991b; Capellades, 1989).

Growth regulators are not directly related to the photosynthetic capacity of cell cultures (Husemann, 1985). However, auxin and ethylene were shown to repress chlorophyll formation (Husemann, 1985) while kinetin is known to be essential for chlorophyll formation (Husemann, 1985).

Therefore, it may be concluded that in vitro plantlets have complete developmemt of the Ps system and may grow autotrophically. However, their Ps

is inhibited by low CO2 concentrations in the vessel and by the presence of sucrose (Kozai, 1991a). The plantlets, thus are forced to grow heterotrophically or mixotrophically by absorbing sucrose from the culture medium as the main carbon source. Even high PPFD was inefficient when CO_2 was limiting in the vessel (Kozai, 1991a). Hence, under limiting carbon dioxide concentrations, plantlets responded to sucrose and failed to grow on sucrose-free media. This explains the frequent earlier failures in inducing autotrophic growth by ommitting sucrose from the medium or increasing light intensity (Kozai, 1991a; Kozai, 1988).

C. Acclimatization

I. Definition

Acclimatization is defined as the process regulated by man to adapt an organism to a new environment (Brainerd and Fuchigami, 1981). In contrast, acclimation is the naturally regulated process by the plant in adapting to the environment (Brainerd and Fuchigami, 1981; Pospisilova *et al.*, 1988). Plantlets in culture were found to acclimate to the specific environment that they grew in (Conner and Thomas, 1981; Pospisilova *et al.*, 1987). The very high relative humidity, low irradiance, heterotrophic or mixotrophic nutrition, high concentration of growth regulators and low gas exchange of the culture vessels resulted in the formation of plantlets with altered morphology, anatomy and physiology which was more typical of shade and hydrophytic plants (Ziv, 1986; Grout and Aston, 1978a; Wetzstein and Sommer, 1983; Smith *et al.*, 1986). These adaptations which were irreversible in most cases (Ziv, 1986; Leshem, 1983a; Donnelly and Vidaver, 1984a; 1984b) made it difficult to successfully transplant them into the more xerophytic ex

vitro conditions without transplant shock which reduced survival drastically (Conner and Thomas, 1981; Pospisilova *et al.*, 1988). It was necessary, therefore, to carefully control hardening-off procedures, 'aclimatization', in order to improve survival upon transfer.

Acclimatization procedures may be divided into two types: a) the classical acclimatization procedures include the ex vitro correction of abnormalities arising from in vitro culture and b) in vitro hardening which includes correction of abnormalities before transplanting to ex vitro conditions.

II. Classical Acclimatization Methods

These procedures depend upon the gradual exposure of IVP to the ex vitro environmental conditions which typically include reduced humidity, elevated light and autotrophic nutrition. Early efforts involved the transfer of the plantlets, on removal from cultural conditions, to an atmosphere of high humidity achieved by a mist sustem (Murashige, 1974; Donnan *et al.*, 1978) or the use of clear plastic covers (humidity tents; Clare and Collin 1974; Sutter and Hutzell 1984; Hasegawa *et al.*, 1973; Conner and Thomas, 1981; Donnan *et al.*, 1978), followed by gradual reduction in humidity and gradual increase in light intensity (Ziv, 1986). Misting had the problem of overwetting plantlets causing fungal decay, reducing O_2 and CO_2 levels, and inhibiting growth (Conner and Thomas, 1981; Clare and Collin, 1974; Donnan *et al.*, 1978; Griffis *et al.*, 1983; Vertesy and Balla, 1987). Humidity tents were also reported to reduce CO_2 concentrations and light in the chambers (Lakso et al, 1986). Others used antitranspirants with limited success (Sutter and Hutzell 1984; Conner and Thomas 1981; Wardle *et al.*, 1979).

Root formation and root quality are critical features for successful transplanting to the greenhouse (McClelland *et al.*, 1990; Mohammed and Donnelly, 1990; Wisniewski *et al.*, 1986). Roots should not be too long before transplanting in order to avoid their breakage (Conner and Thomas, 1981; Maene and Debergh, 1987). In some cases roots were reported to die upon transplanting and new roots form in vivo (Debergh and Maene, 1981; Pierik, 1988). Thus, it was advisable to transplant either when roots were small or immediately after root induction (Conner and Thomas, 1981; Zimmerman *et al.*, 1987; Walker *et al.*, 1987). Differences in root anatomy were reported, with poor vascular system development of in vitro as compared to in vivo plants (McClelland *et al.*, 1990; Smith *et al.*, 1986). In vitro roots lacked root hairs and were not well developed near the soil surface (Mckeand and Wisniewski, 1981; Aldrufeu, 1987). One of the important concerns of inducing roots in vitro is to avoid root development from the callus rather than directly from shoot bases (Hughes *et al.*, 1973; Dunstan, 1981; James and Thurbon, 1979).

Debergh and Maene (1981), Maene and Debergh (1983; 1985) and Donnan *et al.*, (1978) obtained better survival of plantlets by eliminating stage III with direct transplanting of cuttings ex vitro from stage II with rooting in vivo. Since roots produced in vitro were prone to injury upon transplanting and plantlets were reported to undergo cessation of growth until new roots were produced in vivo (Donnan *et al.*, 1978; Maene and Debergh, 1983; Bowden, 1984; Conner and Thomas, 1981; Aldrufeu, 1987; Pierik, 1988; McClelland *et al.*, 1990), rooting in vivo was found to alleviate those problems in several plant species. In vivo rooting was

successful in saving labor costs and producing vigorous plantlets, especially when cuttings were pre-treated with rooting hormones (Debergh and Maene, 1981; Zimmerman *et al.*, 1987; Fabbri and Ranieri, 1987).

Liquid medium overlays used in stage II have also given better results in acclimatization (Maene and Debergh, 1985). They induced vigorous and uniform cuttings in vitro, which enhanced in vivo rooting. In vivo rooting reduced plantlet manipulations and transfer costs. Labor represents approximately 50% of the cost of producing most tissue culture plants with labor for stage III constituting 56 to 75% of the total labor cost (Donnan *et al.*, 1978; Anderson *et al.*, 1977; Fabbri and Ranieri, 1987). Since there is no increase in plantlet number in stage III, the elimination of this stage and the labor involved may lead to substantial savings per unit plant produced (Donnan *et al.*, 1978). However, some plant species do not root in vivo, thus necessitating a preparatory step (stage III) before transplanting (Conner and Thomas, 1981; Bowden, 1984). Rooting in vivo does not eliminate the costs of mists or humidity tents in the greenhouse (Hasegawa *et al.*, 1973).

Elimination of hormones such as auxins and cytokinins in stage III has helped acclimatizing plantlets by allowing better root growth and shoot elongation (Hasegawa *et al.*, 1973; Hasegawa, 1980; James and Thurbon, 1979; Batlle and Aldrufeu, 1987). Rooting at high auxin concentration was reported to produce defective roots which reduced survival of the plantlets (Dunstan, 1981). In addition, the use of hormone-free medium in the rooting stage might reduce deleterious effects of high auxin levels reported to affect the photosynthetic system (Yamada *et al.*, 1978). Reducing salt concentrations to one half or less also helped prepare

plants for autotrophic nutrition (Murashige, 1974; Dunstan, 1981; Hasegawa, 1980; Kozai *et al.*, 1988). Takatori *et al.* (1968) flushed the culture vessels daily for one week with half strength Hoagland solution to slowly promote the switch to autotrophic growth.

Sucrose elimination in stage III was also reported to improve acclimatization (Hasegawa, 1980; Grout and Price, 1978; Kozai *et al.*, 1988). It stimulated a switch to autotrophy. However, sucrose is needed for root initiation in stage III (Rahman and Blake, 1989; Aldrufeu, 1987; Desjardins *et al.*, 1987b).

Non-agar media such as soil mixes (Herman and Hass, 1975; Barnes, 1979; Zimmerman, 1978) vermiculite (Barnes, 1979; Zimmerman, 1978), cotton wool, Jiffy 7 peat pot (Zimmermann, 1987) perlite (Kataeva and Butenko, 1987), sand, cellulose or peat have been used for rooting in stage III or for rooting in vivo (Clare and Collins, 1974; Aldrufeu, 1987; Yang and Clore, 1974). These media often produced a superior root system and extensive root hairs in the species *Citrullus lanatus* L. (Barnes, 1979) and *Malus* sp. (Zimmerman, 1978) as compared to rooting on an agar based-medium. Cauliflower and crysanthemum rooting on cellulose rods (cylindrical plugs of cellulose; Sorbarods, Baumgartner Papier SA, Switzerland) in liquid medium produced an extensive root system in a short time and resulted in superior water uptake as compared to those rooted on agar (Short *et al.*, 1987; Roberts and Smith, 1990). Rooting in vitro shoots on non-agar media proved to be successful for the growth and rooting in culture of cauliflower, African violets, caladium, roses and lilies (Short *et al.*, 1987). In addition, the system

fascilitated the transfer of plantlets and helped to eliminate injury to the roots upon transplanting (Zimmerman, 1978; Short *et al.*, 1987; Yang and Clore, 1974).

Grout and Donkin (1987) noted the importance of studying the in vitro environmental conditions in order to be able to manipulate them in a manner that would allow for in vitro leaves to persist ex vitro and contribute to growth and survival of transplanted plantlets. They suggested three strategies to improve survival ex vitro: 1) maximising leaf growth and thus storage material in vitro, 2) enhancing the develpment of new photoautotrophic leaves ex vitro, 3) altering the in vitro conditions to create photosynthetic capacity in vitro, thereby allowing leaves to persist and support growth upon transfer ex vitro (in vitro acclimatization). Since maximizing leaf growth in vitro may contribute to increased water loss after transfer, enhancing acclimatization ex vitro by improving growth after transplanting was attempted using increased PPFD and CO_2 enrichment in the greenhouse acclimatization conditions. Lakso *et al.* (1986) and Desjardins *et al.* (1987a) increased growth with those treatments, and their plantlets acclimatized earlier. However, despite their treatments, the plantlets required a period of hardening before they regained a balance in their water relations.

III. In Vitro Hardening

The extensive studies of the physiological and anatomical characteristics of IVPs as well as the changes that occurr during acclimatization clarify causes of plantlet death following transplanting. Furthermore, detailed ecophysiological studies led to the conclusion that the in vitro environmental conditions prevailing during the ontogeny of the plantlets were the major cause for the abnormal

behaviour of IVPs upon transplanting. Studies also inferred that "acclimatization" of plantlets was a "developmental" process requiring the development of plantlets under conditions other than the culture environment (Donnelly and Vidaver, 1984b). These conclusions are made from the various relatively irreversible anatomical features such as abnormal leaf anatomy (Leshem, 1983a; b: Donnelly and Vidaver, 1984a; Brainerd *et al.*, 1981; Grout and Aston, 1978a; Ziv *et al.*, 1983) and low epicuticular wax content (Sutter and Langhans, 1979; Grout, 1975), and physiological features such as lack of stomatal functioning (Brainerd and Fuchigami, 1981; 1982; Wardle and Short, 1983) and low photosynthetic rates (Grout and Aston, 1978b; Donnelly and Vidaver, 1984b; Pospisilova *et al.*, 1987) observed.

The high relative humidity, low irradiance, presence of an exogenous carbon source, and low CO_2 concentrations during the ontogeny of the plantlets were the major environmental factors causing the irreversible abnormalities. An aclimatization treatment that changes some of these in vitro environmental conditions could presumably induce morphological, anatomical and physiological developments in plantlets similar to its non-tissue-cultured counterparts. Accordingly, considerable interest has developed in the manipulation of environmental conditions in vitro to effect normal morphogenesis of plantlets (Ziv, 1986; Sutter and Langhans, 1979; Grout and Aston, 1977; Grout and Millam, 1985; Capellades *et al.*, 1990; Smith *et al.*, 1990a; b; Brainerd and Fuchigami, 1981; Donnelly and Vidaver, 1984a; b; Desjardins *et al.*, 1988; Mousseau, 1986; Fujiwara *et al.*, 1987).

Methods used to induce hardening through control of in vitro environmental conditions include: a) relative humidity modifications in vitro, b) Ψ_w modifications of the culture medium, c) plant growth regulators, d) carbohydrate manipulations, e) increasing light intensity, f) increasing CO_2 levels in the gaseous atmosphere, g) automated novel systems.

a. Relative Humidity Modifications

The water-vapor-saturated atmosphere of tissue culture vessels was a primary factor recognized for its effect upon plant morphogenesis in vitro and the resultant poor control over water loss after transplanting (Grout and Aston, 1977; Sutter *et al.*, 1979; Brainerd and Fuchigami, 1981; Wardle *et al.*, 1983a; Ziv, 1986; Ziv *et al.*, 1983). Humidity acclimation refers to the process by which plants grown in high RH adapt to a low RH (Brainerd and Fuchigami, 1981). Attempts, therefore, to reduce the RH in vitro were made and various acclimatization and survival features were examined. Reducing the humidity in vitro was accomplished by: a) uncapping the culture vessels (Brainerd and Fuchigami, 1981); b) using sterilized dessicants such as anhydrous $CaSO_4$ or $CaCl_2$ (Ziv *et al.*, 1983; Sutter and Langhans, 1982), silica gel in the culture vessels or lanolin overlays (Wardle *et al.*, 1983a; Crane and Hughes, 1990); c) increasing the agar concentration of the medium (Ziv *et al.*, 1983; Debergh *et al.*, 1981; Rahman and Blake, 1988); d) cooling the bottom of the culture vessels (Vanderschaege and Debergh, 1987; Maene and Debergh, 1987; Capellades *et al.*, 1990); e) forced ventillation of the culture atmosphere (Walker *et al.*, 1988); f) using lose fitting caps (Maene and Debergh, 1987; Hakkaart and Versluijs, 1983; Short *et al.*, 1987), g) gas permeable caps (Dillen and Buysens,

1989; Kozai *et al.*, 1990; Fari *et al.*, 1987; Short *et al.*, 1987) or containers (Tanaka *et al.*, 1988).

Reducing RH in vitro successfully improved acclimatization of many plant species. Brainard and Fuchigami (1981) obtained significant reduction in water loss of leaves and restored stomatal function in in vitro apple (*M. domestica*) acclimatized by uncapping cultures in the last 4-5 days before transfer. Reducing RH to 35% using lanolin overlays on the medium resulted in increased wax content and reduced water loss in *B. oleracea* (Wardle *et al.*, 1983a) and potato (Solanum tuberosum L. 'Russet Burbank'; Crane and Hughes, 1990), but was detrimental to shoot growth. A similar treatment resulted in reduction of stomatal apertures in *Chrysanthemum xmorifolium*, but high mortality and retardation of stomatal development were observed.

Carnation shoot apices cultured inside a desiccator and exposed to reduced RH developed normal glaucous leaves (Ziv *et al.*, 1983). These plantlets developed surface wax after seven days at 60% and 70% RH, and after five days at 50% RH but not at 80% RH (Ziv, 1986). Survival rates of these acclimatized plants increased from 72% to 90% and 96% after 9 days under 70% and 50% RH, respectively (Ziv, 1986). Increased wax formation was also observed in cabbage plants grown in reduced RH by exposing the cultures to $CaCl_2$ in vitro (Sutter and Langhans, 1982). Fern plants exposed to reduced RH in vitro, survived transplanting to the greenhouse under continuous 80 to 85% RH (Ziv, 1986). Plantlets acclimatized in vitro under 60% or 70% RH for six or nine days showed increased shoot dry weight in the greenhouse.

Ventillation of culture vessels and type of vessel closures were reported to affect vitrification in tissue culture plantlets (Hakkaart and Versluijs, 1983). Short *et al.* (1987) reduced RH in the culture vessel by using various permeable caps. A RH of 80% resulted in normal growth, stomatal function, epicuticular wax deposition and reduced water loss, resulting in successful acclimatization of chrysanthemum (*C. xmorifolium*) and cauliflower (*B. oleracea*) Lower RH of 50% and 30%, however, reduced growth of IVPs (Short *et al.*, 1987).

A lid modified by Dillen and Buysens (1989) to increase evaporation promoted dry weights and reduced vitrification of *Gypsophyla paniculata* L. A thin PVC foil coverage was used by Fari *et al.*, (1987) to overcome acclimatization difficulties. Their treatment resulted in vigorous plantlets with increased dry weight, wax profusion and improved hardening.

Maene and Debergh (1987) activated the sap stream by reducing RH through bottom cooling of the culture vessels. They obtained enhanced shoot quality, wax formation and growth of *Iris germanica* and reduced wilting after transfer to soil in *Calathea ornata.* In a similar way, Capellades *et al.* (1990) reduced RH in cultures of *R. multiflora* to 75% during stage III and demonstrated that epidermal cells and stomata were similar to those of greenhouse leaves.

Tanaka *et al.* (1988) obtained accelerated shoot development and enhanced growth of *Spathiphyllum* plantlets using gas permeable containers 'Culture Pack' made of fluorocarbon polymer films. Smith *et al.* (1990a) reduced the humidity of *Chrysanthemum xmorifolium* cultures to 40% using containers with holes covered by selectively permeable membranes. They obtained plantlets with reduced wilting,

thicker leaves, improved stomatal closure, and increased cuticle thickness, as well as significantly higher chlorophyll concentration.

A semipermeable cover (SPC) developed by Kozai *et al.*, (1988b), to improve gas exchange, particularly CO_2 for photosynthesis, was reported (together with CO_2 enrichment, supplemental lighting, and sucrose elimination) to improve acclimatization of IVPs. The closures enhanced growth of several plant species and were reported to improve acclimatization although actual acclimatization studies were not made (Kozai *et al.*, 1988a; b; Hayashi *et al.*, 1988; Kozai, 1988; Kozai *et al.*, 1990a. A study by Safadi and Hughes (1991) showed that the gas permeable caps caused reduced water loss and increased wax content, as well as reduced wilting injury upon transplanting.

Forced ventilation (Walker *et al.*, 1988) of culture vessels resulted in detrimental effects on plant growth. This was possibly due to the disturbance of the water relations of the plantlets (Kozai, 1991a).

b. Water Potential Modifications

1. Agar

Agar concentration studies were largely done to overcome vitrification which was found to be caused by the high water status of the cultures (Debergh *et al.*, 1981; Arnold and Eriksson, 1983; Ziv *et al.*, 1983). Increasing agar concentration in the culture medium reduced vitrification through a decrease in the matric potential of the medium (Debergh *et al.*, 1981). Agar influence on RH in vitro was shown to be the major controlling factor contributing to vitrification of plantlets (Ziv, 1986).

Raising agar concentrations in the medium to levels of 1.5% to 2% improved epicuticular wax development, induced stomatal function, increased dry weights, chlorophyll concentrations and improved survival in carnation (*D. caryophyllus*; Ziv *et al.*, 1983) as well as normal leaf anatomy in *Picea abies* (L.) Karst. (Arnold and Eriksson, 1984). It also improved survival rates and eliminated vitrification in Globe artichoke (*Cynara scolymus* L. 'Violet d'Huyeres'; Debergh *et al.*, 1981; Debergh, 1983), *P. abies* (Bornman and Vogelmann, 1984), carnation (Leshem, 1983a; b) and quince (*Cydonia oblonga* Mill; Singha *et al.*, 1990). Agar concentrations of 1.4% in the rooting medium increased survival rates of cherry (*P. cerasus*) plantlets from 13% at 0.6% agar to 45% (Marin and Gella, 1987). Higher agar levels, however, drastically reduced shoot multiplication, growth and rooting potential unless the levels were applied after growth had been established (Debergh *et al.*, 1981; Ziv *et al.*, 1983; Arnold and Eriksson, 1983). Normal growth occurred only in the new leaves formed on high agar medium; leaves which had developed on low agar or liquid medium were irreversibly translucent (Ziv *et al.*, 1983; Leshem, 1983a). On the other hand, high agar levels in jackfruit (*Artocarpus heterophyllus* Lam.) cultures reduced RH in culture, but also reduced in vitro rooting and had no effect on the establishment of plantlets in the glasshouse (Rahman and Blake).

The effect of the medium Ψ_w on acclimatization of IVPs was also implicated in studies in which gas permeable caps in culture vessels resulted in increased evaporation rates (Dillen and Buysens, 1989; Maene and Debergh, 1987). Reduction in vitrification of *G. paniculata* was obtained through short-term

reduction in RH (Dillen and Buysens, 1989). These treatments were applied at a critical time of leaf development when plants were found to be susceptible to vitrification-inducing factors. Short-term application was used to avoid the adverse effect of medium drying on growth of the plantlets (Dillen and Buysens, 1989). The increased agar concentration due to evaporation could not entirely account for the reduced vitrification (Dillen and Buysens, 1989). Medium drying caused by losely fit caps also limited the use of such treatment to a period not exceeding 10 days (Maene and Debergh, 1987).

Studies of water relations of tobacco (N. tabaccum L. 'Wisconsin 38') cultures grown in vessels covered with gas permeable caps showed that media Ψ_{ω} was severely reduced (-14 bars) compared to cultures covered with the less permeable B-caps (-4 bars; Safadi and Hughes, 1991). This reduction in Ψ_{ω} could disturb normal water relations of plantlets in vitro.

2. Polyethylene Glycol

Another method for reducing Ψ_{ω} of the medium includes the use of polyethylene glycol (PEG) in the culture medium. This method takes advantage of the properties of PEG which makes it an effective water-stressing agent.

Polyethylene glycol is an inert, nonionic, long chain polymer: $HOCH_2$-(CH_2-O-CH_2)$_x$ CH_2OH (Couper and Eley 1948), available in a range of molecular weights (mw; 200 to 20000), highly soluble in water with low toxicity to mammals (Steuter *et al.*, 1981; Lawlor, 1970). The larger mw forms of PEG do not penetrate into plant cells, as opposed to mannitol and ionic solutes (Thimann *et al.*, 1960; Lagerwerff *et al.*, 1960; Lawlor, 1970). According to Manohar and Heydecker

(1964), PEG of high mw of more than 4000 appear to possess osmotic properties with no evidence of toxicity. Because of these properties PEG has been widely used as a non-penetrating osmoticum to impose water stress on plants (Lagerwerff *et al.*, 1961; Thimann *et al.*, 1960; Janes, 1966; Jackson, 1962; Parmar and Moore, 1968; Kaufmann and Eckard, 1971; Painter, 1966; Heyser and Nabors 1981; Hasegawa *et al.*, 1984).

Lagerwerff *et al.* (1961) used PEG 20,000 to decrease the Ψ_w of kidney bean while Jarvis and Jarvis (1965) applied PEG 1540 to stress tree seedlings. Leshem (1966) grew pine seedlings in PEG 400 and 1500. Janes (1966) reported that bean, pepper, tomato and celery plants grew satisfactorily with no indication of injury in PEG 400, 600, 1000, 1540 and 4000 mw solutions.

Kaufmann and Eckard (1971) reported that PEG 6000 produced changes in plant water relations similar to those caused by drying soil at the same Ψ_w. This was not true for PEG 400 which had detrimental effects on plants. Heysor and Nabors (1981) used PEG 4000 as a non-penetrating osmotic solute to lower the medium Ψ_w of tobacco cell cultures. Similarly, Hasegawa *et al.* (1984) successfully used PEG 6000 to reduce Ψ_w of tomato cell suspension cultures without entry into the cells.

Although PEG has been considered a non-penetrating osmoticum (Hasegawa *et al.*, 1984), there have been strong disagreements about its use to simulate water stress in plants. Several workers reported toxic non-osmotic effects of PEG as a result of either impurities in the chemical or its absorption by plants (Lagerwerff *et al.*, 1961; Greenway *et al.*, 1968; Leshem, 1966; Reid, 1978; Krizek, 1985). Lagerwerff *et al.* (1961) reported toxic effects of PEG 4000, 6000 and 20000 when

applied in the commercial forms, and attributed the toxicity to the presence of large amounts of magnesium and aluminum. This toxicity disappeared after purification. Non-osmotic effects of low concentrations of PEG on roots and root hair elongation of *Agrostis alba* L. were also reported (Jackson, 1962).

An unidentified toxic material (presumably an ionic organic compound) in PEG 1540 inhibited respiration and photosynthesis in *Chlorella pyrenoidosa* (Greenway *et al.*, 1968). Similarly, Parmar and Moore (1968) reported a greater adverse effect of PEG 6000 on corn germination than similar Ψ_w of mannitol and NaCl. Janes (1974) reported toxic effects of nonpurified PEG 4000, 6000 and 1540 resulting from the manufacturing process. Lower mw PEG may also enhance cation accumulation in the root xylem sap to a toxic level (Kaufmann and Eckard, 1971). However, Lawlor, 1970, failed to detect growth or water relations effects in response to purifying PEG. Toxicity was not related to the detergent properties of PEG (Lawlor, 1970).

Toxicity seemed to be associated with various manufacturers' lots, and its effect may be prevented by purification using ion exchange resins, gel filteration or dialysis (Michel, 1966; Lawlor, 1970; Janes, 1974) or by recycling (Plaut and Federman, 1985).

Mexal *et al.* (1975) suggested that the main damage to plants caused by PEG resulted from low oxygen solubility in the root medium, even in dilute PEG concentrations, which restrict root respiration. PEG was also reported to form a boundary layer (Michel, 1971) or a gradient around the root (Kaul, 1966). Thus

stirring or aerating the medium was needed in several studies (Kaul, 1966; Lawlor, 1970; Michel, 1971).

In addition, PEG has been reported to reduce phosphorous uptake (Emmert, 1974) and translocation (Resnick, 1970; Kaufmann and Eckard, 1971), and to cause visible leaf damage (West *et al.*, 1980). Resnik (1970) and Emmert (1974) reported that PEG inhibit translocation of phosphate due to osmotic effects on reducing transpiration and not due to absorbed PEG. In contrast mannitol reduced uptake of phosphates into the plants due to a secondary, non-osmotic effect of mannitol absorbed by the plants (Resnik, 1970). Others, however, reported phosphorous contamination in PEG so that high phosphorous concentrations caused interactions in mineral uptake (Reid *et al.*, 1978).

It is generally believed that PEG molecules with a molecular weight of 3000-4000 or greater do not pass through cell membranes (Michel, 1970; Janes, 1974; Krizek, 1985). However, penetration of PEG into the plant and its non-osmotic effect on plants were reported by several workers (Lagerwerff *et al.*, 1961; Lawlor, 1970; Resnik, 1970; Janes, 1974; Tingey and Stockwell, 1977; Yaniv and Werker, 1983). This presented some doubts on the use of PEG in simulating water stress (Yaniv and Werker, 1983). Lagerwerff *et al.* (1961) detected the presence of unchanged PEG 20,000 on the surfaces of kidney bean leaves. However, they concluded the improbability of a physiological effect of PEG on plants and noted its suitability for use to control osmotic pressure in plants.

Lawlor (1970) showed that healthy intact roots had low permeability to PEG 1000, 4000 and 20,000 and that permeability was accelerated by mechanical damage

of the roots or low Ψ_w. When the high mw PEGs (above 1000) entered plants through injured roots, they caused leaf damages and rapid dehydration (Lawlor, 1970). Lower mw PEG (200) entered the intact roots similarly to mannitol, but their effect on the plant was unlike the higher mw PEGs (Lawlor, 1970). The effect of high mw PEG on plants was suggested to be through blocking water transport pathways (Lawlor, 1970). No effect of PEG on respiration, other than that expected from its osmotic effect, was found. It was concluded that PEGs of large mw were satisfactory to decrease the Ψ_π of nutrient solutions for studies on growth of whole plants as long as the root systems were maintained in a mechanically and physiologically unchanged condition (Lawlor, 1970).

Janes (1974) reported the absorption of small amounts of PEG into roots and its accumulation in leaves of pepper (*Capsicum annum* L. var. California Wonder) plants. The penetration was inversely related to molecular size and directly related to the time of exposure and the decrease in Ψ_π of the nutrient solution. PEG with molecular weights 400 and 600 accumulated in the leaves while a small amount of the higher mw PEG (4000) was absorbed and accumulated in the roots (Janes, 1974). Uptake of PEG into the plant was not related to the rate of transpiration (Janes, 1974).

Resnik (1970) detected uptake of PEG in small amounts by roots of maize plants. Yaniv and Werker (1983) found PEG secretions on leaf surfaces of four solanaceous species when placed on 4.5% solution of PEG 1500, 4000, or 6000, and suggested that PEG should not be used as an osmoticum before its entrance into the plants is tested.

Later reports, however, asserted that PEG was not readily absorbed by plants (Turner and Kramer, 1980; Paleg and Aspinall, 1981). Hasegawa *et al.* (1984) and Heyser and Nabors (1981) report no entry of PEG into the cells of tomato and tobacco in cell suspensions cultures. Insignificant amounts of PEG 4000 were absorbed by *Atriplex canescencs* (Pursh.) Nutt. and *Hilaria jamesii* (Torr.) Benth. (Cress and Johnson, 1987) and *Hordeum vulgare* L. cv. KN 16 (Prasad *et al.*, 1982) as compared to sucrose and mannitol.

PEG continues to be used as an osmoticum in plants to simulate water stress. Since the secreted amounts of PEG on leaves were found unaltered or metabolized (Lagerwerff *et al.*, 1961; Janes, 1974), it was concluded that PEG is inert and does not have metabolic effects on plants other than those due to osmotic stress. The advantages of PEG over mannitol, sucrose or NaCl in inducing water stress includes its nonpenetrability into plants and its lack of metabolization by the plant. It thus provides a constant rate of water stress (Cress and Johnson, 1987; Heyser and Nabors, 1981; Michel, 1970; Slatyer, 1961; Krizek, 1985; Kaul, 1966). However, Krizek (1985) cautioned "one should be aware, the physiological effects due to withholding water and those due to PEG induced water stress may be quite different".

In addition to the use of PEG in reducing Ψ_w of the growth medium, PEG has been used widely in physiological studies of isolated intracellular structures such as organelles and macromolecules (Michel *et al.*, 1983). In plastid isolation, PEG inhibited pigment leakage while in photosynthesis studies PEG increased the activity of the Hill reaction presumably due to its interaction with tannins (Jackson,

1962; Krizek, 1985; Clendenning *et al.*, 1956). PEG is also useful in protoplast preparation, studies of membrane physiology (Benerois and Virville, 1981), measurement of cell wall porosity (Carpita *et al.*, 1979) in transformation and fusion of protoplasts (Money, 1989), and in seed priming to improve rate of germination (Dearman *et al.*, 1986). PEG, as any other osmotic agent, is supposed to control relative humidity in a closed container (Salisbury and Ross, 1985). However, no reports specified the effect of PEG on RH.

Although PEG has been widely used in drought simulation and related studies, the relationship between PEG concentration, molecular weight, and method of determining Ψ_{ω} are not well understood (Krizek, 1985). PEG was found not to conform to Van't Hoff's law, thus measurement of Ψ_{ω} using freezing point depression were not valid (Applegate, 1960; Painter, 1966). Variation of the osmotic coefficient with temperature prevent the use of freezing point depression data at physiological temperatures (Money, 1989; McClendon, 1981).

Lagerwerff *et al.* (1961) reported the solute potential of PEG-Hoagland solutions obtained using freezing point depression methods were 20 to 40% lower than those obtained using thermocouple psychrometer. Steuter (1981) reported the values of Ψ_{π} of PEG 1000, 4000, and 6000 as measured by freezing point depression to be less negative at low concentrations and more negative at higher concentrations. A curvilinear relationship was obtained between molality of PEG solutions and their Ψ_{π} as opposed to true solutions of which Ψ_{π} is a direct function of the number of molecules in solution (Michel and Kaufmann, 1973). Therefore, the concentrations needed to prepare a PEG solution of certain Ψ_{π} were

determined graphically for each mw PEG using thermocouple psychrometers (Lagerwerff *et al.*, 1961; Lawlor, 1970; Williams and Shaykewich, 1969; Michel and Kaufmann, 1973; Steuter *et al.*, 1981; Money, 1989). Regression equations of the relationship of PEG concentration with Ψ_w were also formulated (Michel and Kaufmann, 1973; Michel, 1983; McClendon, 1981). These relationships show a change in molecular configuration of PEG with concentration (Michel and Kaufmann, 1973) and the number of molecular subunits (ethylene oxide subunits). The molecular configuration relate to the water bonding, and not the number of particles (polymer chains), which determine the Ψ_w of solutions (Steuter *et al.*, 1981; Money, 1989). This suggests that the effect of PEG on Ψ_w is through matric potential and should be referred to as "matricum" rather than osmoticum (Steuter *et al.*, 1981).

It has been reported that PEG solutions of high mw and in concentrations used in physiological experiments behave like colloids, and matric forces are a major component of the resulting Ψ_w (Steuter *et al.*, 1981). Therefore, psychrometric values represent the best available guide to the total osmotic effect of PEG in solution (Michel and Kaufmann, 1973; Steuter *et al.*, 1981; Money, 1989).

Viscometry using refractive index offers a simple, rapid and accurate method for measuring PEG content of solutions (Michel and Kaufmann, 1973; Lagerwerff *et al.*, 1961). One of the factors which may also cause errors in calculating Ψ_w of PEG solutions is PEG interactions with the ionic nutrient solutions mixed with it so that synergism occurs (Lagerwerff *et al.*, 1961; Michel and Kaufmann, 1973; Money, 1989). Since most PEG studies occur in nutrient solutions, care should be

taken not to calculate Ψ_w in the experimental medium by adding the Ψ_w of each constituent (Money, 1989).

The effect of PEG on plants were mostly the same as those of water stress. Reduction of plant growth (Jarvis, 1963; Kaul, 1966; Jackson, 1962; Slatyer, 1961; Ciamporova and Luxova, 1975; Kawasaki *et al.*, 1983) and Ψ_w of the plants (Jarvis, 1963; Prasad *et al.*, 1982; Lawlor, 1970; Kaufmann and Eckard, 1971), osmotic adjustment (Turner and Kramer, 1980; Janes 1966) and reduced transpiration and nutrient uptake (Resnik, 1970; Lawlor, 1970; Janes 1966; Kawasaki *et al.*, 1983) were effects reported for PEG. Low turgor causes slowing or cessation of growth as a result of effects on protein synthesis, cell wall synthesis, and membrane production (Acevedo *et al.*, 1971). Indeed, the first influence of water stress on plants is known to be reduction in growth (Hsiao and Bradford, 1983; Salisbury and Ross, 1985). However, some plants are capable of osmotic adjustment which maintains growth at certain degrees of water stress or reduction in Ψ_w (Turner and Kramer, 1980; Janes, 1966). Induction of or selection for drought resistance in plants (Kaul, 1966; Turner and Kramer, 1980) and cell cultures (Heyser and Nabors,1981; Hasegawa *et al.*, 1984; Handa *et al.*, 1982; Bressan *et al.*, 1981) has also been done through the use of PEG simulation to water stress. Water stress preconditioning has also induced drought resistance characteristics such as reduced transpiration, increased epicuticular wax content, reduced leaf size and increased root shoot ratios (Levit, 1980; Seiler, 1985; Kummerow, 1980).

In plant micropropagation, PEG use has been limited to few uses in acclimatization where it is used to precondition plantlets to water stress before

transfer ex vitro (Kandeel and Hughes, personal communication, Zaid, 1990; Safadi *et al.*, 1990; Short *et al.*, 1987). PEG treatment in stage III has been reported to reduce water loss from detached leaves, to increase leaf surface wax contents in microprpagated date palms (Phoenix dactylifera) (Zaid, 1990) and to induce stomatal closure in in vitro tobacco (*N. tabaccum*; Safadi, 1992). It has also been reported to increase wax content in cauliflower (*B. oleracea*; Short *et al.*, 1987), to decrease water loss and to induce normal leaf anatomy in grapevines (Dami and Hughes, 1991a; b).

The beneficial effect of low Ψ_w due to PEG was offset, however, by the deleterious effect on growth (Safadi, 1992; Kandeel and Hughes, personal communication; Short *et al.*, 1987; Dami and Hughes, 1991a). The entry of PEG into the roots through damaged roots was reported to cause severe reduction in growth (Lawlor, 1970). PEG interference with the delicate water relations of the plantlets may also cause undesired effects (Kozai, 1991a).

Water stress by agar (Ziv *et al.*, 1983) or reduced RH (Smith *et al.* (1990a) as well as results from tobacco plantlets growing under reduced RH induced by gas permeable caps (Safadi *et al.*, 1991) caused increased chlorophyll concentrations. Chlorophyll could have increased due to the increased gas exchange and resulting Ps activity in cultures covered with semipermeable caps. However, it could also have been the result of higher light intensity or even a change in the light quality due to caps. It is known that light quality affects chlorophyll synthesis (Husemann, 1985). It is also known that the development of chlorophyll is antagonistic with growth (Huseman, 1985; Hughes, 1981). Since water stress caused reduced growth,

it is possible that reduction in growth due to water stress led to an increase in chlorophyll. The method of measuring chlorophyll also is different when on a leaf area basis as compared to fresh or dry weight basis since unexpanded leaves would have larger amounts of chlorophyll per unit area (Bjorkmann and Holmgren, 1963). However, chlorophyll content was not found to be correlated to Ps capacity (Huseman, 1985; Bjorkman and Holmgren, 1963).

Ionic salts such as NaCl, $CaCl_2$ or KNO_3 were also used as osmotica in the study of water relations of plants (Slatyer, 1961; Jensen, 1981). It has been shown that ionic salts penetrate into the plants and thus may interfere with their normal metabolism (Levitt, 1980; Kaul, 1966; Parmar and Moore, 1968; Slatyer, 1961). The effect of ionic salts such as NaCl, on growth of plants has been shown to be due to a) osmotic stress and b) specific ion effect on metabolic processes due to absorption of NaCl and c) the nutritional imbalances caused by these ions (Parmar and Moore, 1968; Kingsbury and Epstein, 1986; Kawasaki *et al.*, 1983; Janes, 1970). High concentrations of NaCl have been shown to inhibit the absorption of ions such as K^+, Ca^{+2} and Mg^{+2} to a higher degree than PEG (Kawasaki *et al.*, 1983). High concentrations of Na^+ and Cl^- ions could be detrimental to metabolic processes, such as enzyme activity, protein synthesis, nitrogen absorption and assimilation and photosynthesis (Binzel *et al.*, 1985). However, the presence of calcium in the medium was reported to alleviate the toxic effects of Na^+ (Cramer *et al.*, 1987) and enhance normal stomatal functioning in in vitro plantlets (Ziv *et al.*, 1987). In tobacco seedlings, increasing NaCl concentrations caused a remarkable increase in leaf succulence indicated by lower ratio of dry weights to fresh weights (Flowers *et*

al., 1986). The Na:Ca^{++} ratio was shown to have a profound effect on succulence and the transport properties in the roots (Flowers *et al.*, 1986). Calcium was reported to be important for counteracting the effect of Na^+ in plants and maintaining normal permeability of cell membranes (Cramer *et al.*, 1987).

Water stress induced by either PEG or NaCl salt was found to induce osmotic adjustment (reduction of sap Ψ_π) of the plants which allowed them to maintain leaf turgor while Ψ_ω decreased (Janes, 1966; Kawasaki *et al.*, 1983, Kaufmann and Eckard, 1971; Jensen, 1981). Osmotic adjustment was reported to occur due to accumulation of Na^+, and Cl^- ions in NaCl osmotica treatments while PEG-treated plants accumulated organic solutes such as carbohydrates (Janes, 1966; Slatyer, 1961). However, gradual application of PEG was essential since abrupt exposures to low Ψ_π caused wilting and injury of the plants (Janes, 1966).

The use of PEG and NaCl as water stress simulators was reported to equally affect turgidity, Ψ_ω and growth of plants (Janes, 1966). However, others found larger effect of PEG on growth of plants than NaCl, but NaCl inhibited ion uptake more than PEG (Janes, 1966; Kawasaki *et al.*, 1983). Water stress induced by salts resulted in faster osmotic adjustment of plants grown due to NaCl absorption (Slatyer, 1961; Janes, 1966; Jensen, 1981). Osmotic adjustment induced by pre-conditioning can reduce the severe effects of low Ψ_ω in the root medium and is widely used for hardening plants against water stress (Levitt, 1980; Jensen, 1981).

However, it is not known how this pre-conditioning would harden tissue culture plantlets. Due to the high RH in their growing conditions, tissue culture plantlets are known to have delicate water relations (Kozai, 1991a). It has been

reported that plants grown under high humidity with PEG in the medium undergo guttation when penetrating PEG 400 was used but not the PEG 6000 (Kaufmann and Eckard, 1971).

c. Plant Growth Regulators

Growth regulators during plantlet growth and morphogenesis are important in determining root and shoot quality and their competency for survival ex vitro. Cytokinins stimulate chlorophyll formation and protein synthesis which affect assimilation (Farquhar and Sharkey, 1982). While cytokinins are necessary for shoot proliferation and auxins for root initiation, introducing a transfer stage onto hormone-free medium, before or after root initiation caused shoot growth and improved shoot quality (Maene and Debergh, 1983; 1985; 1987; Debergh and Maene, 1981; Batlle and Aldrufeu, 1987; Conner and Thomas, 1981).

Ethylene accumulation and its retroinhibition effect on lignification has also been implicated in causing vitrification. Ventillating culture vessels, therefore was suggested to prevent ethylene retroinhibition (Kevers *et al.*, 1984; Hakkaart and Veersluijs, 1983; Walker *et al.*, 1988). However, other studies could not relate vitrification to ethylene accumulation in the culture vessel (Debergh *et al.*, 1981). In another study ethylene was suggested to promote dry weights and number of shoots (Walker *et al.*, 1988).

Growth retardants seemed to be a promising method in in vitro acclimatization (Ziv, 1986). However, various combinations of a number of auxins and several growth retardants have been found ineffective in reducing vitrification in carnation and artichoke plantlets (Ziv *et al.*, 1983; Leonhardt and Kandeler,

1987; Debergh *et al.*, 1981). Abscissic acid was ineffective as an antitranspirant in *B. oleracea* (Wardle *et al.*, 1979) and *M. domestica* (Brainerd and Fuchigami, 1982). On the other hand, rooting of *Asparagus officinalis* L. 'Jersey Centennial' was superior and survival was improved when an antigiberellin "ancymidol" was used for rooting (Desjardins *et al.*, 1987b; Chin, 1982). In vitro *Asparagus* also responded to B-995, phosphon, amo 1618, cycocel, and paclobutrazol with stronger shoot and root development (Khunachak *et al.*, 1987).

Prunus avium formed shorter stems, darker leaves and survived better in cold storage after treatment with paclobutrazol (Snir, 1988). A recent report showed a positive hardening effect of the plant growth retardant paclobutrazol when included in the rooting medium of *Chrysanthemum xmorifolium* plantlets (Smith *et al.*, 1990b). This treatment reduced wilting, induced smaller stomatal apertures, increased epicuticular wax, shortened stems and thickened roots which may have subsequently reduced water loss.

d. Carbohydrate Level in the Medium

The use of increased concentrations of sucrose and mannitol in the culture media as osmotica for the reduction of vitrification in carnation (Ziv *et al.*, 1983) and globe artichoke (Debergh *et al.*, 1981) was unsuccessful. However, sucrose function in heterotrophic growth of plantlets implies a different perspective to its role in acclimatization. The ommission of sucrose from the medium in stage III has been suggested as a method to induce autotrophic growth of plantlets (Grout and Donkin, 1987; Grout and Price, 1987). The previous authors succeeded in inducing autotrophic growth on newly-formed leaves under sucrose-free medium.

However, several others failed to induce autotrophic growth in plants after ommision of sucrose which was indispensible for growth (Evers, 1982; Langford and Wainwright, 1987; Solarova, 1989). Kozai *et al.* (1988a; b) were able to induce autotrophy in vitro upon ommission of sucrose only when other conditions for photosynthesis such as CO_2 and light levels were optimized. They suggest that the ommission of sucrose in the acclimatization stage was an essential part of their proposed method of acclimatization (Kozai, 1988; Kozai, 1989).

On the other hand, sucrose was found to be essential for root initiation in many plant species (Desjardins *et al.*, 1987b; Hyndman *et al.*, 1983; Rahman and Blake, 1988). Root formation and quality are crucial for the successful micropropagation and survival of plantlets, since they support the plantlet while it is being acclimatized in the greenhouse. Since root formation depends upon an endogenous carbohydrate level (Rahman and blake, 1988), the supply of light and sucrose, their proper timing and their effect on various plant species need to be considered in planning for in vitro acclimatization.

e. Light Intensity

In earlier sections of this review, light intensity was shown to be essential for the formation of leaf surface wax and normal leaf anatomy. It is also involved in stomatal function and photosynthetic activity. Increasing light intensity in stage III was attempted to correct for the features contributing to their poor survival upon transplanting (Donnelly and Vidaver, 1984a,b; Wetzstein and Sommer, 1983; Lee *et al.*, 1985; 1988). However, it was soon realised that light was ineffective under low CO_2 concentrations in the culture vessels (Kozai, 1988; Kozai, 1991a; Kozai *et*

al., 1990a; Kozai *et al.*, 1988a). Increasing light intensity was then incorporated into integrated acclimatization methods to induce autotrophic growth with sucrose ommission, increased PPFD, and CO_2 enrichment (Kozai, 1988; Kozai, 1991a; Hayashi *et al.*, 1988; Fujwara *et al.*, 1988).

f. Carbon Dioxide Enrichment

CO_2 enrichment after transfer of IVPs to the greenhouse has been attempted by a few researchers to achieve rapid growth and high survival rates of the plantlets during acclimatization (Lakso *et al.*, 1986; Fujiwara *et al.*, 1987; Kozai *et al.*, 1987a). Lakso *et al.* (1986) promoted growth of non-rooted transplanted shoots 20 and 30 days after transplanting. Similar treatments by Desjardins *et al.* (1987a) promoted growth of strawberry plantlets ex vitro and shortened the period needed for the plantlets to reach acclimatization. However, neither addressed the critical water stress period immediately after transfer.

Kozai *et al.* (1987a) and Hayashi and Kozai (1987) developed an acclimatization unit with controlled environment ([CO_2], PPFD, RH, and temperature) for transferring plantlets during acclimatization under non-sterile conditions. They obtained improvement in the survival rates from 80% to 96% in strawberry and from 78% to 92% in taro. Similar to previous work by Lakso et al. (1986) and Desjardins et al. (1987) the CO_2 enrichment in the acclimatization unit shortened the acclimatization period but the improvement in growth was delayed, after 28 days, and was insufficient to significantly reduce post-transplanting water stress. Moreover, the system needed acclimatization curves established for each plant species (Hayashi and Kozai, 1987); Kozai *et al.*, 1987c). Since acclimatization

of IVPs needs to develop during the ontogeny of plantlets, applying acclimatization treatments to plantlets mature enough to be removed from culture was questionable (Walker *et al.*, 1988). In addition, these systems did not eliminate the costly handling and treatments in the greenhouse.

The finding that tissue culture plantlets had photosynthetic ability and that low CO_2 concentrations in culture vessels was the major limiting factor to Ps led to CO_2 enrichment studies under in vitro conditions. Plantlets were found to develop photautotrophy provided that physical environmental factors such as CO_2, light etc. in the vessel were properly controlled (Fujiwara *et al.*, 1987). CO_2 enrichment was attempted in what was termed the preparation stage: a stage between the propagation stage and the acclimatization stage (Kozai and Iwanami, 1988). These studies combined light intensity enhancement (2 to 3 times higher PPFD than standard in vitro conditions), sucrose removal, as well as nutrient salts and vessel stopper modifications.

Carbon dioxide enrichment of tobacco plantlets increased Ps considerably with more significant effect when sucrose was ommitted (Mousseau, 1986). Increasing PPFD and improving gas exchange of culture tubes, in the preparation stage, increased the net Ps and chlorophyll content of strawberry plantlets but did not enhance growth (Kozai and Sekimoto, 1988). They suggested the need for further improvement in the environment (presumably CO_2 levels) of tissue culture vessels (Kozai and Sekimoto, 1988).

Carbon dioxide enrichment of 950 ppm during the light period under relatively high photon fluxes (150 μmole $m^{-2}sec^{-1}$) increased growth of statice

(*Limonium* Hybrid) plantlets in vitro (Kozai *et al.*, 1987b). Similarly, CO_2 enrichment, high PPFD and a relatively high gas infiltration rate promoted photosynthetic rates and growth of chlorophyll containing *Cymbidium* 'Reporsa' plantlets (Kozai, *et al.*, 1987c) carnation (Kozai and Iwanami, 1988; Kozai *et al.*, 1988b) and potato (Kozai *et al.*, 1988a). In in vitro *Rosa hybrida* Hort. 'Mary Antwennett', CO_2 enrichment was more effective on sucrose-free medium and high PPFD than sucrose-containing medium at low light levels (Kozai *et al.*, 1990a). Increased PPFD was ineffective in promoting Ps under low CO_2 concentrations (Kozai and Sekimoto 1988; Kozai, *et al.*, 1988a; Kozai *et al.*, 1990a). *Cymbidium* 'Reporsa' plantlets grown under CO_2 gave photosynthetic response curves relating net photosynthetic rates, PPFD and CO_2 concentrations which were similar to those for *Cymbidium* plants grown in vivo (Kozai. *et al.* 1990b).

A gas semi-permeable transparent plastic cap (SPC) was devised (Kozai *et al.*, 1988b) to increase gas infiltration of the culture vessels. The cap consisted of two kinds of films: one was a transparent polymethylpentene film (brand name: TPX film, Mitsui Petrochemical Co.), the other was a microporous polypropylene film (Brand name: Celgard 4410, Hoecht Calanese Co.). A hole (8mm diameter) was made at the center of the square TPX film and was covered with the Celgard film (10 mm in diameter). A solvent (Toluene 30%, xylene 15% and MIBK 5%) was used to glue the two films (Kozai *et al.*, 1988b). The SPC resulted in 5.6 to 7.6 air changes per hour as compared to an aluminum foil cap (0.4) and plastic formed cap (1.5). The SPC was expected to provide for higher CO_2 levels in in vitro vessels

than in the air-tight vessels, maintain lower RH in the vessel, and provide higher PPFD level than the aluminum cap (Kozai *et al.*, 1990a).

In the absence of gas permeable closures and CO_2 enrichment, however, plantlets needed sucrose to grow, since low CO_2 concentration limited Ps and growth (Kozai and Iwanami, 1988; Solarova, 1989; Kozai *et al.*, 1988a). Forced ventillation into the culture vessels was not successful in improving growth rate of tissue cultured *Rhododendrum* 'P.J.M.'. This treatment may have interfered with the balance of gases in the vessels (Walker *et al.*, 1988) or disturbed the delicate water relations of plantlets (Kozai, 1991a).

Successes were obtained in achieving photoautotrophic growth in vitro in chlorophyll-containing micropropagated shoots or plantlets by applying conditions favorable for photosynthesis. Conditions included CO_2 enrichment, high PPFD, high gas exchange stoppers and little or no sucrose. Phoptoautotrophic tissue culture had important implications for developing a new mass propagation system offering remarkable advantages in minimizing costs of sucrose and contaminations. Advantages of introducing photoautotrophic growth in tissue culture include: 1) reduction of cost associated with reduced need for of sucrose, 2) elimination of contamination risk, 3) ability to use larger vessels and non-sterile conditions, 4) development of a shorter culture and acclimatization period since growth is enhanced, and 5) the application of automation or robotization to save labor (Kozai and Iwanami, 1988; Kozai, 1988). In addition, larger, more vigorous plants could be more adaptable to transplanting, resulting in improvement of survival of in vitro plantlets upon transfer to ex vitro conditions.

g. Novel Automated Systems

Based upon the substantial advantages obtained from the autotrophic system as noted previously, a new approach to micropropagation emerged called "sugar free micropropagation" or "photoautotrophic tissue culture system (PTCS)". It has been applied to automated systems in large containers (Fujiwara *et al.*, 1988; Aittken-Christie and Davis, 1988).

A prototype of the novel tissue culture system was developed by Fujiwara *et al.* (1988) and described by Kozai *et al.* (1987a), Hayashi and Kozai (1987) and Hayashi *et al.* (1988) to culture shoots and/or plantlets photoautotrophically. The system consists of a computerized, labor saving, automated assembly of a large culture box, gas flow devices and a culture solution assembly with a support mmaterial such as rockwool. Shoots or plantlets are grown under sucrose-free conditions, CO_2 enrichment and high PPFD, a low relative humidity and non-aseptic conditions (Fujiwara *et al.*, 1988; Kozai *et al.*, 1987a; Hayashi and Kozai, 1987).

The PTCS system caused substantial enhancements in growth, as measured in dry weights, fresh weights and relative growth rates, as well as enhanced net Ps rates in strawberry (Kozai *et al.*, 1987; Fujiwara *et al.*, 1988) carnation (Hayashi, *et al.*, 1988) and *Cymbidium* (Kozai *et al.*, 1987c) plantlets. The growth promotion and the control of the environmental conditions under PTCS was presumed to lead to acclimatization of plantlets; hence, the system was also referred to as an "acclimatization unit" (Hayashi *et al.*, 1988; Kozai *et al.*, 1987).

The system was later found to be well-suited for propagation of small explants (Hayashi *et al.*, 1988; Kozai *et al.*, 1990b). Explants of single node

carnation with 2 leaves (Hayashi *et al.*, 1988) and *Cymbidium* (Kozai *et al.*, 1990b) were grown into more vigorous plantlets in the acclimatization unit than explants grown conventionally. The daily growth of these plantlets was greater than conventionally-grown plantlets, although it was growing autotrophically; no sugar was added. The system was adaptable to further optimization such as decreased O_2, use of a hydroponic nutrient solution and lowered night temperature to increase net daily photosynthesis.

The PTCS was presumed to improve survival of plantlets since it enhanced the factors which would help the plantlets tolerate water stress at transplanting. These included plantlet vigor, promotion of root development and increased dry weight (Kozai, 1988; Hayashi *et al.*, 1988). These features were reported to improve the transplantability of CO_2 enriched *Liquidambar* seedlings (Tolley and Strain, 1984a, b). Carnation plantlets grown in the PTCS were more vigorous with a higher growth rate and had leaves which were larger in size, darker green, and thicker than those micropropagated conventionally (Hayashi *et al.*, 1988). Anatomical features such as increased epicuticular wax and improved stomatal functioning were assumed to be enhanced under the high light and low humidity conditions of PTCS (Hayashi *et al.*, 1988).

Although the PTCS promoted growth of strawberry, little work other than growth studies were done on the actual acclimatization and survival of the autotrophic plantlets upon transfer ex vitro. A rather crude experiment of strawberry and taro plantlets acclimatized in the acclimatization unit showed reduced death rates upon transfer (4% and 8%) as compared to traditionally

acclimatized plantlets (20% and 23% respectively; Hayashi and Kozai, 1987; Kozai *et al.*, 1987a). The photoautotrophic system proved to increase growth and vigor of potato plantlets, but did not reduce the water loss of detached leaves (Fujiwara *et al.*, 1988). This indicated that solving for autotrophy did not necessarily solve for the problem of water stress that plantlets experience upon transfer to ex vitro conditions.

Reduction of RH in the acclimatizing unit was recommended to induce water stress hardiness into the tissue culture plantlets (Fujiwara *et al.*, 1988). The relationship between autotrophy and acclimatization is still not clearly elucidated. Desjardins *et al.*, (1988) reported that treatments (CO_2 enrichment and high PPFD) which induced autotrophy in plants improved growth but did not obviate the necessity for an acclimatization treatment to habituate plantlets to low humidity. Their preliminary results, however, detected increased stomatal frequency with increasing autotophy, but failed to detect significant anatomical changes that would lead to successful acclimatization to low humidity.

The use of gas permeable caps in combination with high PPFD and CO_2 enrichment was effective in promoting growth of shoots and roots of potato (Kozai *et al.*, 1988a), chrysanthemum (Kozai, 1988) carnations (Kozai *et al.*, 1988b) and roses (Kozai *et al.*, 1990a). Plastic formed lids with relatively reduced gas exchange (1.5) promoted little increase in growth rates inspite of CO_2 enrichment (Kozai *et al.*, 1987b). Under low CO_2, SPC (Number of air changes 3.8) promoted an increase in dry and fresh weights and Ps of *Rosa* plantlets over cultures with aluminum closures. However, larger enhancements in Ps (2 times) and growth were

obtained when combined with sucrose-free conditions. CO_2 concentrations were 470 ppm in SPC-covered vessel compared to 190 ppm in Aluminum-foil capped vessels (Kozai *et al.*, 1990a).

Although the effect of the gas permeable cap on Ps was evaluated, the RH in SPC covered vessels and its influence upon plantlet response to water stress was not measured. The effect of the gas permeable cap on the water relations of plantlets in relation to their acclimatization as well as evaluation of PTCS system for acclimatization are areas which warrant further work.

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CHAPTER III

EFFECT OF PEG AND SALT OSMOTICA ON MOISTURE LOSS AND GROWTH OF IN VITRO TOBACCO PLANTLETS

A. Introduction

Poor survival of in vitro plantlets (IVP) after transplanting is a problem for commercial micropropagation of many plant species. Plants grown under in vitro conditions often undergo a transplant shock characterized by uncontrolled water loss and desiccation when transferred to ex vitro conditions (Grout and Aston, 1977; Wardle *et al.*, 1979; Brainerd and Fuchigami, 1981). Problems associated with transplanting IVPs have been largely attributed to lack of epicuticular wax (Grout and Aston, 1977; Sutter and Langhans, 1979; 1982; Wardle *et al.*, 1979), abnormal stomatal function (Brainerd and Fuchigami, 1981; 1982; Wardle and Short, 1983) abnormal leaf internal and surface anatomy (Donnelly and Vidaver, 1984; Brainerd *et al.*, 1981; Wetzstein and Sommer, 1982; Fabbri *et al.*, 1986), and poor root-shoot vascular connections (Grout and Aston, 1977).

Many tissue culture plants were reported to experience water loss exceeding 50% in the first 15 to 60 minutes after transplanting (Brainerd and Fuchigami, 1981; Conner and Conner, 1984; Pospisilova *et al.*, 1987; Dhawan and Bhojwani, 1987). This has been found to be detrimental to cells of 'Pixy' plum as measured by electrolyte leakage, ethylene and ethane emissions (Kobayashi *et al.*, 1981).

Methods to reduce water loss in IVPs include those which induce normal wax deposition, stomatal function, and leaf and root anatomy. Exposure of cultures to reduced relative humidity, increased light intensity and reduced medium water potentials (Ψ_{ω}) during the acclimatization stage (stage III), were among the most successful hardening procedures (Ziv, 1986; Brainerd and Fuchigami, 1981).

Reduction of medium Ψ_{ω} through increased agar concentrations produced normal glaucous carnation (*Dianthus caryophyllus*; Ziv *et al.*, 1983), *Gypsophyla* (Dillen and Buysens, 1989) and artichokes (Debergh *et al.*, 1981), increased wax deposition and normal stomatal functioning in carnation (Ziv *et al.*, 1983; 1987) and cauliflower (*Brassica oleracea*; Wardle *et al.*, 1983). The agar effect on acclimatization has been shown to be the result of its effect on RH in culture vessels (Ziv, 1986; Rahman and Blake, 1988) and on reducing the water status of the plantlets (Ziv *et al.*, 1983; Ziv, 1986). In these studies, however, significant reduction in growth and multiplication occurred which offset the beneficial effect of increased agar concentration on acclimatization (Debergh *et al.*, 1981; Wardle *et al.*, 1983; Ziv *et al.*, 1983).

Polyethylene glycol (PEG) as an osmotic agent which simulates water stress has been widely used in water stress and drought adaptation studies (Hasegawa *et al.*, 1984; Jackson, 1962; Janes, 1966; Jensen, 1981; Krizek, 1985; Lawlor, 1970). In micropropagation, PEG added to the culture medium increased wax formation in *Chrysanthemum xmorifolium* and cauliflower (*B. oleracea*) but reduced growth substantially (Short *et al.*, 1987). PEG as a supplement to the rooting medium was found to reduce water loss of detached leaves and to increase wax deposition in

vitro potato (*Solanum tuberosum* L. 'Rrusset Burbank'; Kandeel and Hughes, unpublished results), date palms (*Phoenix dactylifera* L.; Zaid and Hughes, 1989), and grape (*Vitis vinifera* L. 'Valiant'; Dami, 1991). In addition, stomatal closure was reported in PEG-treated tobacco (*Nicotiana tabaccum* L. 'Wisconsin 38'; Safadi *et al.*, 1990). However, growth was significantly reduced in both grape (Dami and Hughes, 1991a) and potato (Kandeel and Hughes, unpublished results).

The effect of PEG on plant growth has been largely attributed to its osmotic effects on plants. Higher molecular weights of PEG were shown to cause greater reduction in growth than the lower molecular weight due to the their greater effect on Ψ_w (Kawasaki *et al.*, 1983, Janes, 1974). PEG-induced water stress decreased growth (Janes, 1966; Jensen, 1981; Prasad *et al.*, 1982; Kawasaki *et al.*, 1983), reduce transpiration (Janes, 1966; Michel, 1970); and decrease uptake of minerals such as K^+, Ca^{++}, Mg^{++} (Kawasaki *et al.*, 1983) and phosphorous (Resnik, 1970).

PEG has been considered a non-penetrating osmoticum (Hasegawa *et al.*, 1984). However, several workers cautioned against the toxic effect of PEG due to its penetration or toxic contaminants (Lagerwerff *et al.*, 1961; Greenway *et al.*, 1968; Leshem, 1966; Reid *et al.*, 1978; Krizek, 1985). It is generally believed that PEG molecules with a molecular weight of 3000-4000 or greater do not pass through cell membranes (Michel, 1970; Janes, 1974; Krizek, 1985). However, Lawlor (1970) showed that PEG 4000 penetrated damaged and broken roots, thus the importance of using undamaged roots in PEG stressing experiments (Krizek, 1985). PEG was also reported to reduce the solubility of oxygen in the root medium (Mexal *et al.*, 1975) and to form a boundary layer (Michel, 1971) or a gradient around the root

(Kaul, 1966). Stirring or aerating the medium overcame this problem (Kaul, 1966; Lawlor, 1970; Michel, 1971).

Ionic salts such as NaCl, $CaCl_2$ or KNO_3 were also used as osmotica in the study of water relations of plants (Slatyer, 1961; Jensen, 1981). Ionic salts do penetrate into plants and thus may interfere with their normal metabolism (Levitt, 1980; Kaul, 1966; Parmar and Moore, 1968; Slatyer. 1961). The effect of ionic salts such as NaCl, on growth of plants have been shown to be due to a) osmotic stress b) specific ion effect on metabolic processes due to absorption of NaCl and c) the nutritional imbalances caused by such ions (Parmar and Moore, 1968; Kingsbury and Epstein, 1986; Kawasaki *et al.*, 1983; Janes, 1970).

High concentrations of NaCl have been shown to inhibit the absorption of ions such as K^+, Ca^{+2} and Mg^{+2} to a greater degree than PEG (Kawasaki *et al.*, 1983). High concentrations of Na^+ and Cl^- ions also could be detrimental to metabolic processes, such as enzyme activity, protein synthesis, nitrogen absorption and assimilation and photosynthesis (Binzel *et al.*, 1985). The presence of calcium in the medium was reported to alleviate the toxic effects of Na^+ (Cramer *et al.*, 1987) and enhance normal stomatal functioning in vitro plantlets (Ziv *et al.*, 1987). In tobacco seedlings, increasing NaCl concentrations caused a remarkable increase in leaf succulence as indicated by a lower ratio of dry weights to fresh weights (Flowers *et al.*, 1986).

The Na:Ca ratio has profound effect on the transport properties of the roots (Flowers *et al.*, 1986). Increasing the Na:Ca ratio by decreasing Ca^{++} concentration increased the accumulation of Na^+ and Cl^{-1} into the leaf tissue and increased

succulence. Chloride ion concentrations were found to be linearly related to succulence in tobacco tissues. Calcium was reported to be important for counteracting the effect of Na^+ in plants by maintaining normal permeability of cell membranes (Cramer *et al.*, 1987).

Water stress induced by either PEG or salts was found to induce osmotic adjustment (reduction of sap osmotic potential [Ψ_π]) of plants. This allowed them to maintain leaf turgor while Ψ_w decreased (Janes, 1966; Kawasaki *et al.*, 1983, Kaufmann and Eckard, 1971; Jensen, 1981). Osmotic adjustment was due to accumulation of Na^+ and Cl^- ions in NaCl osmotica treatments while PEG-treated plants accumulated organic solutes such as carbohydrates (Janes, 1966; Slatyer, 1961). However, gradual application of PEG was essential since abrupt exposures to low Ψ_π caused wilting and injury of the plants (Janes, 1966).

The use of PEG and NaCl as water stress simulators was reported to equally affect turgidity, Ψ_w and growth of plants (Janes, 1966). However, others found a greater reduction of growth with PEG than NaCl, although NaCl inhibited ion uptake more than PEG (Janes, 1966; Kawasaki *et al.*, 1983). Stress induced by salts resulted in faster osmotic adjustment of plants due to NaCl absorption (Slatyer, 1961; Janes, 1966; Jensen, 1981).

Osmotic adjustment induced by pre-conditioning (pre-exposure to water stress) can reduce the severe effects of low Ψ_w in the root medium and is widely used for hardening plants against water stress (Levitt, 1980; Jensen, 1981). However, it is not known how this pre-conditioning would harden tissue culture plantlets. Tissue culture plantlets have delicate water relations due to their highly

humid growth conditions (Kozai, 1991). Plants grown under high humidity with PEG in the medium exhibited guttation when penetrating PEG 400 was used (Kaufmann and Eckard, 1971).

In this report several, concentrations of PEG and a mixture of equal amounts of the ionic salts NaCl and $CaCl_2$ were evaluated for their effect on acclimatization of tobacco plantlets, as evaluated by moisture loss of detached leaves and relative wax deposition. The effect of the treatments on growth of the plantlets is also assessed in an attempt to find a suitable method of in vitro acclimatizing tissue culture plantlets.

B. Materials and Methods

Tobacco (*Nicotiana tabaccum* L. 'Wisconsin 38') plantlets were grown from single nodal sections on agar solidified (0.6%) Murashige and Skoog medium (MS; Murashige and Skoog, 1962) with 3% sucrose and no hormones in baby food jars (200 ml) capped with B-caps (Magenta Corp., Chicago). Culture conditions were $25 \pm 3^{\circ}C$ with continuous light supplied by cool fluorescent lights at 80 $\mu mol\ m^{-2}s^{-1}$ PPFD. Tobacco seedlings were grown in a greenhouse in 15 cm clay pots with soil mix of 2 peat : 1 vermiculite : 2 perlite (by volume) watered with 20N-10P-20K Peters fertilizer. Plants were grown under natural photoperiod and light in the greenhouse, which ranged between 300 to 800 $\mu mole\ m^{-2}\ s^{-1}$ PPFD. Temperatures and RH in the greenhouse ranged between 20 to 27° C and 20% to 90% respectively. The humidity during most of the growth period ranged between 50 and 70% RH. Plants were grown to the same physiological stage of in vitro plantlets indicated by the number of leaves.

Plantlets at the 7 to 8 leaf stage were transferred into PEG or salt supplemented liquid medium (as above) and were supported by filter paper bridges in baby food jars (200 ml). Supplements of PEG (6000-8000 mw) at concentrations of 0.0%, 1.0%, 2.5%, 5.0%, 10.0%, 15.0%, or 20.0% (weight per volume of solution) were added and incubated for 3, 6, and 10 days. Salt concentrations of 0.0%, 0.5%, 1.0%, or 1.5%, [equal amounts (by weight), of NaCl and $CaCl_2$] were also used for 3, 6, and 10 days.

Water potential of the medium with PEG was measured using thermocouple psychrometers in a C-52 sample chamber (Wescor Inc., Logan, Utah) connected to a computerized microvoltmeter (Model HP-115, Wescor Inc, Logan, Utah). Water potential of various concentrations of PEG are given in Table 3.1.

Moisture loss of detached leaves (abaxial side up), exposed to ambient laboratory conditions of about 20% RH, 21° C and 10 μmole m^{-2} s^{-1} light, was measured as an index of acclimatization. Samples from PEG, salt and greenhouse treatments were weighed at 0, 15, 30, 60, 120 and 240 minutes. Dry weights were determined by drying for 48 hours at 70°c in a drying oven.

Moisture loss/unit dry weight was determined using the following formula:

$$\frac{(fwt_o - dwt) - (fwt_t - dwt)}{dwt}$$

where fwt_o = fresh weight at zero time, fwt_t = fresh weight after time, dwt = dry weight. Moisture loss on a dry weight basis has been found to be more reliable in monitoring moisture loss of plants than on fresh weight basis (McCaig and Romagosa, 1989). Percent moisture loss (% ML) has also been calculated by the

Table 3.1. Water potential of ms culture medium supplemented with various levels of polyethylene glycol (mw 6000-8000; PEG) with or without agar or agar + PEG.

Agar	% PEG	Water potential bars
0.0	0.0	-4.003
0.0	1.0	-4.373
0.0	2.5	-5.034
0.0	5.0	-5.218
0.0	10.0	-6.480
0.0	15.0	-8.003
0.0	20.0	-11.614
0.6	0.0	-4.019
0.6	0.25	-4.381
0.6	0.5	-5.231
0.6	1.0	-6.149
0.6	1.5	-6.663

following formula: $$\frac{(fwt_o-dwt)-(fwt_t-dwt)x100}{(fwt_o-dwt)}$$

Rate of moisture loss (RML) was calculated from the amount of water per unit dry weight lost in four hours.

Leaf epicuticular wax was quantified by extracting waxes from detached leaves dipped for 3 consecutive dips in warm (40° C) chloroform. The chloroform was then pooled and evaporated using a rotovaporizer (R-Bushi, Rinco Instrument CO.). The last aliquots of chloroform with the dissolved wax were poured into pre-weighed aluminum dishes and evaporated under a hood. The wax content was determined gravimetrically and expressed on a leaf area basis. Leaf area was

measured before extraction using a LI-COR area meter (model LI-3100, Lambda Inst. Corp. Lincoln, Nebraska).

Fresh weight, number of leaves, stem length, leaf area, leaf dry weight and root dry weight, all taken at the time of water loss measurement.

In all of the above measurements, ten replicates per treatment were used in a completely randomized design. Analysis of variance and mean separation were done using General Linear Models and Student-Neuman-Keul's mean separation method (SAS, 1985).

The relationship between RML and wax content for the treatments 0%, 1.0%, 2.5%, and 5.0% PEG and greenhouse seedlings, was estimated by a polynomial regression of RML versus wax and wax^2. Separate intercepts in the regression equation for PEG plants and greenhouse plants were allowed for by inclusion of an additional independent variable taking the value of zero for the PEG-treated plants and one for greenhouse plants. The coefficient associated with the additional variable estimates the difference in average RML between PEG and greenhouse plants at the same wax content.

C. Results

I. Experiment 1. Effect of salts on

a. Water loss. Equal amounts of ionic salts ($NaCl$ + $CaCl_2$) osmotica were tested in various concentrations and durations for their effect on water loss of detached leaves of IVPs. Salt treatments reduced water loss per unit dry weight of detached leaves, especially when used in higher concentrations of 1.5% and 2.0% and for the longer duration (Fig. 3.1d,e,f). The lower concentrations

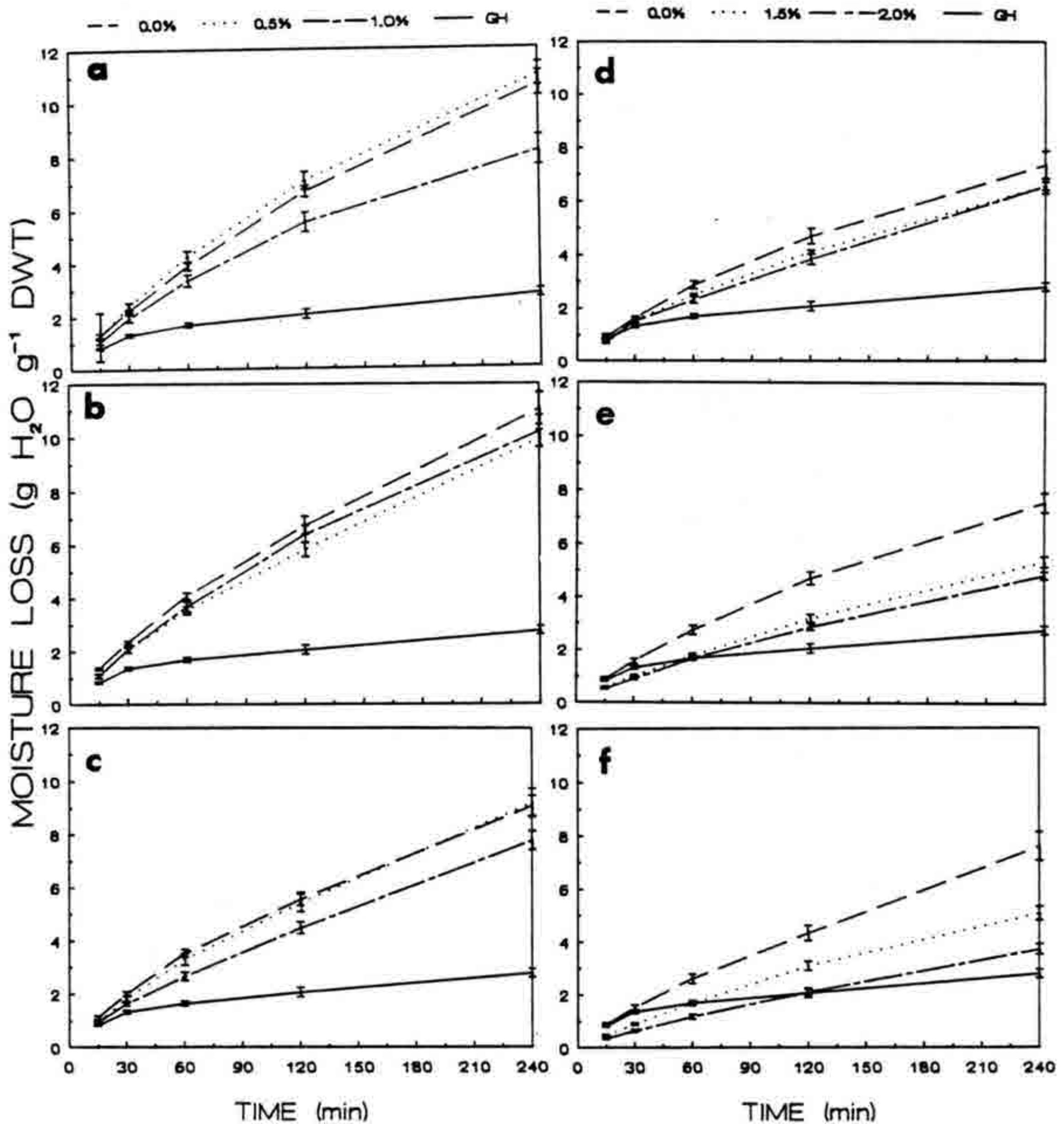

Figure 3.1. Moisture loss per unit dry weight (Dwt) of in vitro plantlets treated with 0, 0.5%, 1.0% NaCl+$CaCl_2$, for **a)** 3 days, **b)** 6 days **c)** 10 days, and 1.5% and 2.0% NaCl + $CaCl_2$ for **d)** 3 days, **e)** 6 days and **f)** 10 days. Moisture loss for greenhouse (GH) plants is included for comparison. Vertical bars represent ± standard error.

(0.5% and 1.0% salts) and shorter of treatments for the 1.5% and 2.0% salts (3 days) had little or no effect on reducing water loss of detached leaves (Fig. 3.1a,b,c,d). Salt concentrations of 1.5 and 2.0% reduced % ML at 4 hour, of detached leaves (Table 3.2) by 14.4% and 8.5% respectively compared to that that of control when plantlets were treated for 3 days; 34% and 29.4% when treated for 6 days; and 29% and 40% when treated for 10 days (Table 3.2).

The rate of moisture loss per unit dry weight per hour was also reduced in a similar manner. Significant reductions over control were only caused by 1.5 and 2.0% salt concentrations applied for 6 and 10 days (Table 3.2). However, the percentage loss of water content by the end of 4 hours of drying was still higher (23% to 29%) than that of the GH plants (20%). The RML at those treatments (1.270 g H_2O g Dry wt^{-1} h^{-1} and 0.0926 g H_2O g Dry wt^{-1} h^{-1}) was also higher than that for the greenhouse plants (0.695 g H_2O g Dry wt^{-1} h^{-1}; Table 3.2).

Although 1.5 and 2.0% salts had water loss in the range of that for the GH plants at the beginning, water loss continued to increase while greenhouse plants' water loss levelled off after 30 minutes (Fig. 3.1). The higher concentrations of salts, however, gave rise to succulent growth with increased water content and increased growth of leaves and stems, as will be shown in the growth data. The leaf area was enlarged and leaves were chlorotic with lower leaves necrotic at the higher concentrations and durations of treatments. Chlorosis was similar to mineral deficiency symptoms.

Table 3.2. Percent moisture loss (% ML) and rate of water loss (RML; gH_2Og dry weight^{-1} h^{-1}) of tobacco plantlets treated with NaCl + $CaCl_2$ at 0.0%, 0.5%, 1.0%, 1.5% and 2.0% for 3, 6, or 10 days. % ML of greenhouse plants (GH) is included for comparison.

Salt[1] % Treat-ment	Days	% ML after time (min) 15	30	60	120	240	RML $g\ g^{-1}\ h^{-1}$
0.0	3	6.26 a[z]	10.71 a	18.76 a	32.10 a	50.95 a	2.657 a
0.5	3	5.89 a	11.52 a	20.03 a	33.16 a	51.60 a	2.747 a
1.0	3	6.23 a	11.42 a	19.61 a	32.35 a	47.64 a	2.033 b
0.0	6	10.08 a	11.34 a	19.81 a	32.55 a	53.22 a	2.764 a
0.5	6	5.09 b	9.77 a	16.92 a	27.75 a	47.07 a	2.485 a
1.0	6	4.83 b	9.35 a	16.64 a	29.13 a	6.27 a	2.555 a
0.0	10	5.53 a	9.98 a	17.69 a	27.93 a	45.41 a	2.271 a
0.5	10	4.16 a	8.17 b	14.83 b	24.83 a	41.44 a	2.199 a
1.0	10	4.86 a	8.62 ab	13.91 b	23.62 a	41.09 a	1.945 a
0.0	3	5.48 a	9.90 a	17.36 a	28.25 a	43.91 a	1.835 a
1.5	3	5.23 a	8.87 a	14.28 b	23.58 b	37.61 a	1.638 a
2.0	3	4.16 a	9.27 a	14.15 b	23.40 b	40.18 a	1.637 a
0.0	6	5.11 a	9.13 a	16.01 a	27.48 a	44.21 a	1.897 a
1.5	6	2.96 b	5.48 b	9.55 b	17.27 b	29.01 b	1.344 b
2.0	6	3.27 b	5.76 b	10.79 b	18.48 b	31.20 b	1.215 b
0.0	10	4.37 a	7.68 a	13.38 a	22.26 a	39.24 a	1.896 a
1.5	10	2.58 b	5.01 b	9.30 b	17.06 b	27.92 b	1.270 b
2.0	10	2.18 b	4.07 b	7.42 c	13.28 c	23.49 b	0.926 c
GH		5.97	9.63	12.32	15.03	19.98	0.695

[z]Mean separation using Student-Newman-Keules Test. Means followed by the same letter do not differ significantly at the 0.5 level of significance.

[1]NaCl + $NaCl_2$

b. Growth. Salt treatments (NaCl + $CaCl_2$) of 0%, 0.5%, and 1.0% did not reduce growth (Table 3.3). On the contrary, a concentration of 1.0% salt increased fresh weights, dry weights, stem length, and number of leaves significantly over that of 0.5% salt and control. Root dry weight did not show statistical differences although there was some reduction in dry weights of roots due to the salt treatment (Table 3.3). Likewise, concentrations of 1.5% and 2.0% NaCl increased growth characteristics significantly over control (Table 3.4). However, number of leaves was not affected indicating greater expansion of leaves. Root dry weight was reduced significantly at the longer incubation period of 10 days.

II. Experiment 2. Effect of PEG in solid medium on

a. Water loss. Plantlets were grown from single node explants for 32 days on agar-solidified medium supplemented with concentrations of 0.25%, 1.0% and 1.5% PEG. A significant reduction in water loss per unit dry weight was also observed throughout the drying period (Fig. 3.2). Water loss per unit leaf dry weight at the end of the 4-hour-drying period was reduced by 31%, 73% and 62% for treatments 0.25%, 1.0% and 1.5% respectively.

b. Growth. Direct culture of plantlets on solid media supplemented with 1.0 or 1.5% PEG significantly reduced growth for all parameters examined (Table 3.5). PEG concentrations of 0.25%, 1.0% and 1.5% inhibited the growth and rooting in about 30%, 90% and 89% of the cultures respectively (Fig. 3.3). The severe reduction in growth and lack of rooting limited the use of this procedure for hardening in vitro plantlets.

Table 3.3. Effect of 0.0%, 0.5%, 1.0% (NaCl + $CaCl_2$) on growth of tobacco plantlets grown for 3, 6 and 10 days.

% salt	days	Fwt[1] (g)	Dwt[2] (g)	stem length (cm)	No. of leaves	root Dwt (g)	H_2O content (g)	Dwt/ Fwt
0.0	3	1.64 a[z]	0.075 b	6.1 a	9.1 a	0.030 a	1.57 a	0.05 b
0.5	3	2.03 a	0.092 ab	5.4 a	9.4 a	0.022 a	1.93 a	0.05 b
1.0	3	2.01 a	0.115 a	7.4 a	10.0 a	0.019 a	1.90 a	0.06 a
0.0	6	2.72 b	0.13 b	14.22 a	14.00 a	0.035 a	2.60 a	0.05 a
0.5	6	3.23 b	0.14 b	11.20 ab	12.75 a	0.034 a	3.10 a	0.05 a
1.0	6	4.85 a	0.21 a	10.07 b	12.29 a	0.024 a	4.50 a	0.04 a
0.0	10	2.52 b	0.12 b	15.76 a	12.29 b	0.043 a	2.40 b	0.05 a
0.5	10	4.21 a	0.18 a	15.58 a	14.22 a	0.049 a	4.00 a	0.04 a
1.0	10	3.72 a	0.18 a	9.17 b	11.83 b	0.025 a	3.50 a	0.05 a

[z]Mean separation using Student-Newman-Keuls Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

[1]Fwt = Fresh Weight

[2]Dwt = Dry Weight

Table 3.4. Effect of 0.0%, 1.5% and 2.0% salt (NaCl + $CaCl_2$) on growth of tobacco plantlets grown for 3, 6 and 10 days.

% salt	days	Fwt[1] (g)	Dwt[2] (g)	stem length (cm)	No. of leaves	root Dwt (g)	H_2O content (g)	Dwt/ fwt
0.0	3	1.72 b[z]	0.10 b	5.9 a	9.9 a	0.034 a	1.63 b	0.06 a
1.5	3	2.70 a	0.15 a	4.9 a	9.4 a	0.030 a	2.64 a	0.05 a
2.0	3	3.20 a	0.18 a	4.8 a	10.3 a	0.031 a	3.00 a	0.06 a
0.0	6	2.30 b	0.13 b	7.7 a	12.4 a	0.110 a	2.21 b	0.06 ab
1.5	6	4.30 a	0.22 a	5.7 a	12.5 a	0.040 a	4.14 a	0.05 b
2.0	6	4.70 a	0.27 a	5.1 a	11.2 a	0.050 a	4.40 a	0.06 a
0.0	10	3.00 b	0.15 b	7.1 a	13.2 a	0.060 a	2.83 b	0.05 b
1.5	10	4.80 a	0.24 a	5.6 ab	14.2 a	0.040 b	4.52 a	0.05 b
2.0	10	4.50 a	0.27 a	4.8 b	11.3 a	0.030 b	4.20 a	0.06 a

[z]Mean separation using Student-Newman-Keuls Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

[1]FWT = Fresh Weight

[2]DWT = Dry Weight

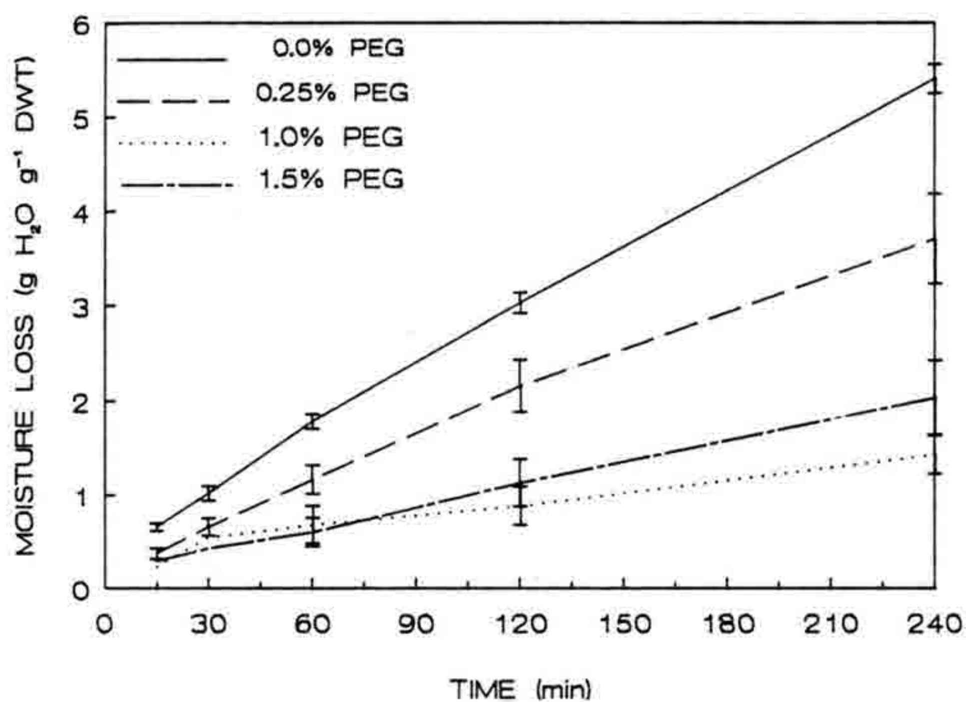

Figure 3.2. Water loss per unit dry weight (g H_20 g^{-1} DWT) of detached leaves from in vitro plantlets grown on solid medium with 0.0%, 0.25%, 1.0%, 1.5% polyethylene glycol (PEG) for 32 days.

Table 3.5. Effect of 0, 0.25, 1.0% and 1.5% PEG + on tobacco plantlets grown for 32 days in agar solidified medium.

% PEG	Fwt[1] (g)	Dwt[2] (g)	Dwt/ Fwt (g)	leaf area (cm^2)	stem length (cm)	No. of leaves (g)	Root Dwt (g)
0.0	2.186 a[z]	0.143 a	0.066 b	75.16 a	4.26 a	8.22 a	0.003 a
0.25	2.295 a	0.153 a	0.077 b	89.00 a	5.36 ab	8.67 ab	0.073 a
1.0	0.501 b	0.055 b	0.177 a	8.32 b	2.57 bc	7.20 bc	0.029 b
1.5	0.472 b	0.062 b	0.189 a	15.78 b	1.89 c	6.30 c	0.024 b

[z]Mean separation using Student-Newman-Keuls Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

[1]Fwt = Fresh Weight

[2]Dwt = Dry Weight

Figure 3.3. Tobacco plantlets grown on agar solidified (0.6%) medium supplemented with 0, 0.25, 1.0% and 1.5% polyethylene glycol (PEG) showing the effects of PEG on growth of plantlets.

III. Experiment 3. Effect of 10%, 15%, and 20% PEG on

a. Water loss. Detached leaves from in vitro plantlets (control; 0.0% PEG) rapidly lost water upon air drying under laboratory condition.Control plantlets lost about 50% of their water content in the first two hours after detachment (Table 3.6). By the end of four hours the leaves had lost 55 to 75% of their water content. In contrast, leaves from the greenhouse (GH) grown seedlings lost only 20% of their water content after four hours (Table 3.6). Leaves from vitro plantlets wilted in the first 30 minutes by becoming flaccid, rolling their edges in one hour, and desiccating in 3 to 4 hours (Fig. 3.4).

PEG in various concentrations and durations of treatment were tested. A choice of the most suitable osmoticum concentration and duration of application depended upon its effect on reducing water loss of the detached leaves and on growth characteristics. PEG used in concentrations of 10.0%, 15.0% and 20.0% for 3, 6 and 10 days of incubation caused severe wilting of the leaves. Wilting started from a few hours to 24 hours after treatment and developed into injury by the third day with desiccation by the sixth day. The treated plants, however, did not die. The apex gave rise to new leaves which were reduced in size. The higher concentrations combined with longer treatment gave rise to smaller and fewer leaves (1 to 3 leaves at most).

Table 3.6. Percent moisture loss (% ML) of tobacco plantlets treated with 0%, 10% and 15% Polyethylene glycol (mw 6000 - 8000; PEG) for 3 days as well as 20.0% PEG for 3, 6 and 10 days, and greenhouse (GH) plants.

PEG %, Treat- ment	Days	% ML after time (minutes)				
		15	30	60	120	240
00.0%	3	12.0 a[z]	21.1 a	33.4 a	54.6 a	76.5 a
10.0%	3	10.4 a	17.2 a	27.0 b	40.3 b	55.6 b
15.0%	3	11.7 a	20.2 a	30.1 ab	45.6 b	60.3 b
00.0%	3	8.6 a	15.6 a	26.7 a	43.2 a[1]	64.2 a[2]
20.0%	3	6.8 b	12.5 b	21.6 b	36.8 a	56.3 a
00.0%	6	10.9 a	13.1 b	23.9 a	38.7 a	58.5 a
20.0%	6	11.6 a	19.5 a	28.0 a	45.5 a	68.1 a
00.0%	10	12.3 a	20.5 a	32.3 a	52.6 a	70.1 a
20.0%	10	12.7 a	20.9 a	34.7 a	51.4 a	67.3 a
GH		6.00	9.6	12.3	15.0	20.00

[z]Mean separation using Student-Newman-Keules Test. Means followed by the same letter do not differ significantly at the .05 level of probability (P).

[1]P=0.0536

[2]P=0.0504

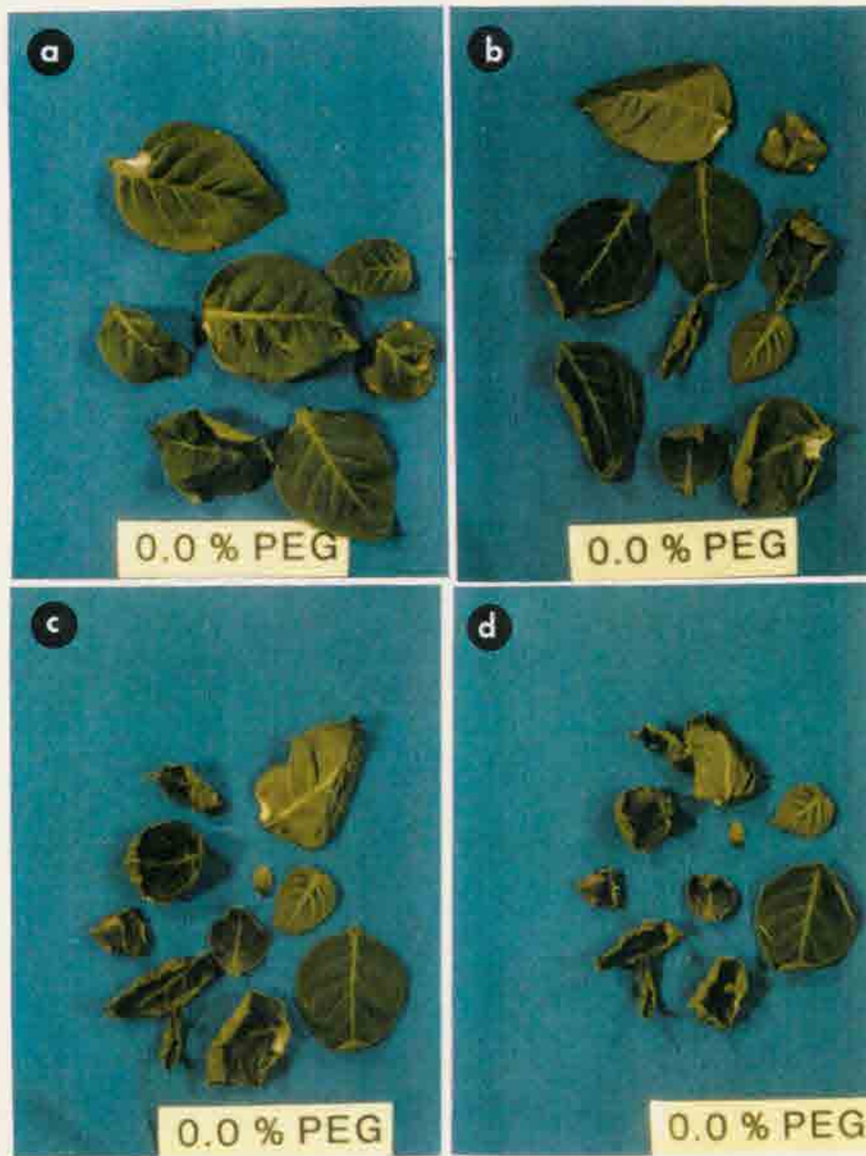

Figure 3.4. Leaves of in vitro tobacco plantlets (IVPs) at **a)** time of detachment (0 time), **b)** 1 hour, **c)** 2 hours and **d)** 4 hours after detachment.

Treatments with 10%, 15% and 20% PEG for 3 days reduced moisture loss by 27%, 21% and 12% respectively over that of the control after 4 hours of detachment (Table 3.2). However, the % ML was still high and leaves were losing more than 50% of their water content. Treating the plantlets with 20% PEG for 6 or 10 days did not reduce % ML of detached leaves (Table 3.6). This was likely due to the injurious effect of the treatment. The longer treatments (10 and 15% PEG for 6 and 10 days) also caused severe injury to the plant. Reduction in % ML in leaves derived from plantlets treated with 0, 10 and 15% PEG for 3 days could be associated with newly-formed leaves under the milder stress treatments. The new leaves in the latter treatments were small, thick, leathery and remained rigid throughout the drying period. These results suggest that lower concentrations of PEG are more suited since they had less leaf injury than higher concentrations and allowed for the formation of new leaves.

b. Growth. Plantlets grown on the high concentrations of PEG (10%, 15%, and 20% PEG) indicated significant reductions in fresh weight, dry weight, leaf area, stem length and number of leaves (Table 3.7). Root dry weight, however, was not affected.

These reductions were observed for all concentrations and incubation times of 3, 6 and 10 days (Table 3.7). Data for growth of tobacco plantlets preconditioned by 10% and 15% for 10 days was not obtained due to the severe wilting of the plants.

Table 3.7. Effect of PEG 0, 10%, 15% and 20% levels on growth parameters of tobacco plantlets treated for 3 and 6 days.

% PEG	Days	Fwt[1] (g)	Dwt[2] (g)	leaf area (cm^2)	stem length (cm)	No. of leaves	root Dwt (g)
00.0	3	1.69 a[z]	0.12 a	67.9 a	2.7 a	11.0 a	0.050 a
10.0	3	0.54 b	0.08 b	26.5 b	1.9 b	8.5 b	0.040 a
15.0	3	0.45 b	0.07 b	24.0 b	1.8 b	8.2 b	0.030 a
00.0	6	---	0.19 a	102.5 a	3.5 a	11.3 a	0.065 a
10.0	6	---	0.11 b	19.3 b	2.0 b	8.3 b	0.062 a
15.0	6	---	0.11 b	17.7 b	1.6 b	8.3 b	0.045 a
00.0	3	1.35 a[z]	0.07 a	49.4 a	3.90 a	8.27 a	0.029 a
20.0	3	0.65 b	0.06 a	28.2 b	2.60 b	6.60 b	0.033 a
00.0	6	1.35 a	0.09 a	53.9 a	5.22 a	10.40 a	0.038 a
20.0	6	0.52 b	0.07 b	20.5 b	3.03 b	7.00 b	0.036 a
00.0	10	1.85 a	0.16 a	79.9 a	4.16 a	10.70 a	0.057 a
20.0	10	0.52 b	0.09 b	27.5 b	1.74 b	8.09 b	0.056 a

[z]Mean separation using Student Newman Keuls Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

[1]Fwt = Fresh Weight

[2]Dwt = Dry Weight

IV. Experiment 4. Effect of 1.0%, 2.5%, and 5.0% PEG on

a. Water loss. Polyethylene glycol, used in liquid medium in lower concentrations than those presented in Table 3.6, was investigated for its effect on hardening and growth of in vitro tobacco plantlets. Concentrations of 1.0%, 2.5% and 5.0% PEG reduced % ML, RML (Table 3.8) and water loss per unit dry weight (Fig. 3.5a,b,c). Only concentrations of 2.5% and 5.0% PEG reduced % ML to levels below 50% after 4 hours of desiccation (Table 3.8). None of the treatments reduced % ML to levels comparable to greenhouse plants (20%; Table 3.8). Rate of water loss on a dry weight basis was reduced by all treatments with the lowest rates obtained with 2.5% and 5.0% PEG for 10 days. These rates (0.685g g dwt^{-1} h^{-1} and 0.490 g g dwt^{-1} h^{-1}) were comparable to those obtained from greenhouse plants (0.695 g g^{-1} dwt h^{-1}; Table 3.9).

A 10 day PEG treatment of 2.5% and 5.0% reduced water loss per unit dry weight to levels comparable to greenhouse (Fig. 3.5c). Water loss for PEG treated leaves continued to increase while greenhouse moisture loss leveled off after 30 minutes.

Treatments with these lower concentrations of PEG (1.0, 2.5, and 5.0%) caused wilting of the lower leaves. This wilting was more severe at 2.5%, and 5.0% PEG with the 10-day-treatment. However, new leaves developed quickly. These leaves were slightly reduced in size and appeared thick and leathery. Upon detachment, they remained turgid even after 4 hours of treatment as compared to control plants which were desiccated (Fig. 3.6, 3.7, 3.8).

Table 3.8. Percent moisture loss (% ML) of tobacco plantlets treated with 0.0 %, 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for 3, 6 and 10 days.

PEG % Treat-ment	Days	% ML after time (min) 15	30	60	120	240
0.0	3	12.87 a[z]	21.80 a	34.48 a	49.93 a	70.04 a
1.0	3	10.56 ab	17.13 a	26.56 b	42.75 b	65.82 a
2.5	3	8.31 ab	15.17 a	24.41 b	38.10 b	58.35 b
5.0	3	9.88 b	20.00 a	27.02 b	41.97 b	61.56 ab
0.0	6	13.94 a	23.45 a	38.59 a	57.16 a	75.97 a
1.0	6	11.93 a	19.94 ab	30.52 b	43.98 b	59.73 b
2.5	6	10.47 a	18.31 b	27.10 b	40.64 b	53.42 bc
5.0	6	12.04 a	17.63 b	26.40 b	37.77 b	50.17c
0.0	10	12.78 a	21.29 a	33.58 a	55.85 a	71.66 a
1.0	10	8.10 b	12.78 bc	22.04 b	37.13 b	55.30 b
2.5	10	6.46 b	10.28 c	18.32 b	30.18 b	44.05 b
5.0	10	9.57 b	15.93 b	24.43 b	35.39	49.75 b
GH plants		5.97	9.63	12.32	15.03	19.98

[z]Mean separation using Student Newman Keules Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

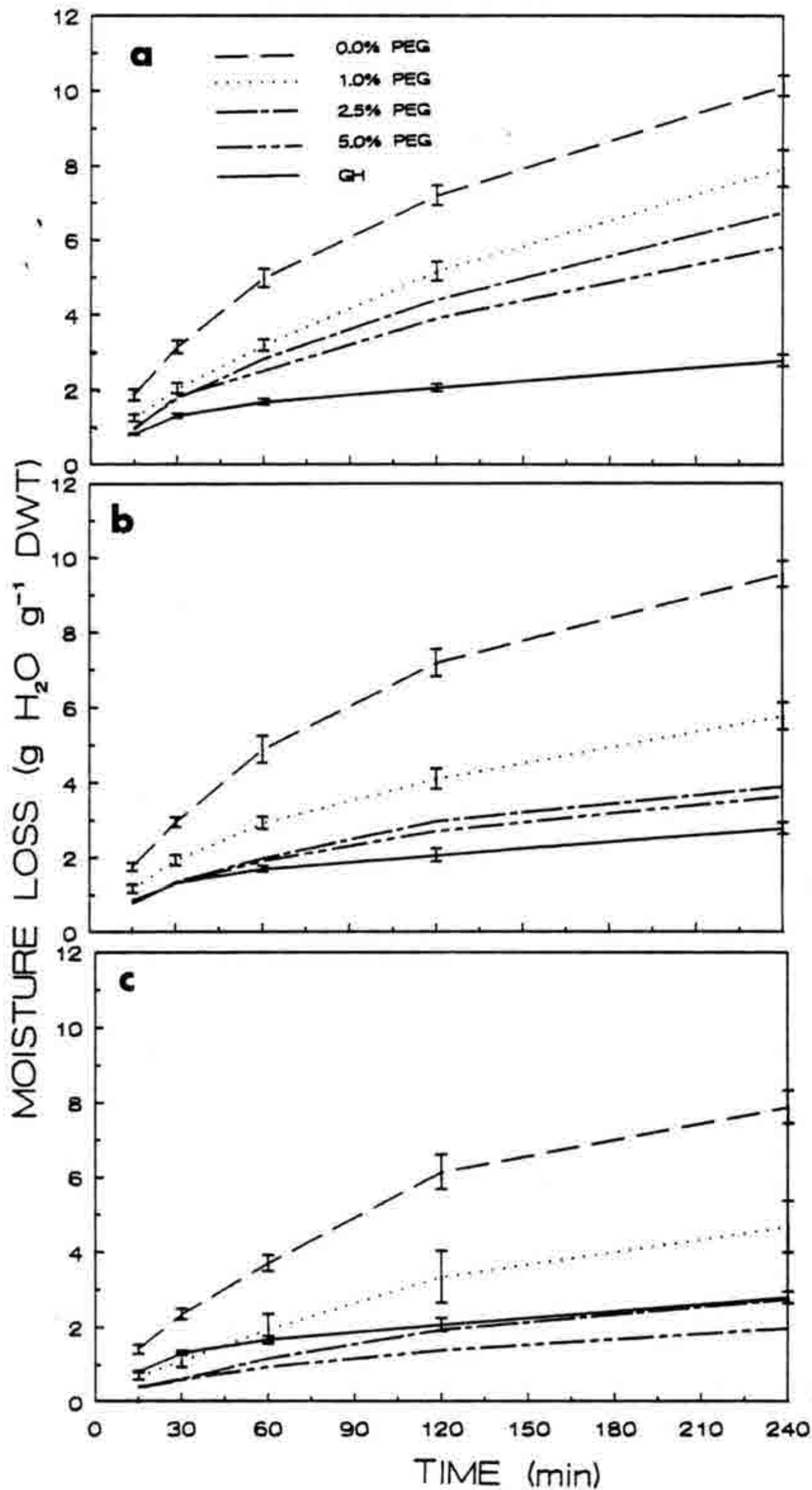

Figure 3.5. Water loss per unit dry weight (g H_2O g^{-1} DWT) of in vitro tobacco plantlets treated with 0.0% 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for **a**) 3 days **b**) 6 days **c**) 10 days. Greenhouse (GH) plant water loss is included for comparison. Vertical bars represent $\pm$ standard error. Bars are omitted on graphs where overlap occurs.

Table 3.9. Percent moisture loss (% ML), rate of moisture loss (RML; g H_2O g^{-1} dry weight h^{-1}) and wax content of tobacco plantlets treated with 0.0 %, 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for 3, 6 and 10 days.

PEG % Treat-ment	Days	RMl $g\ g^{-1}\ h^{-1}$	WAX $mg\ cm^{-2}$	Dwt^2/Fwt^1
0.0	3	2.529 a	0.089 b	0.065 d
1.0	3	2.021 b	0.161 b	0.078 c
2.5	3	1.685 bc	0.321 a	0.082 b
5.0	3	1.456 c	0.444 a	0.101 a
0.0	6	2.385 a	0.095 c	0.074 c
1.0	6	1.440 b	0.226 c	0.096 b
2.5	6	0.976 c	0.384 b	0.123 a
5.0	6	0.911 c	0.568 a	0.130 a
0.0	10	1.970 a	0.069 a	0.084 c
1.0	10	1.170 b	0.268 c	0.113 bc
2.5	10	0.685 c	0.547 b	0.143 b
5.0	10	0.490 c	0.874 a	0.221 a
GH plants		0.695	0.069	0.068

Mean separation using Student-Newman-Keule's Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

[1]Fwt = Fresh Weight

[2]Dwt = Dry Weight

Figure 3.6. Detached leaves of in vitro tobacco plantlets grown with 0% (control), 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for 3 days, **a)** at time of detachment and **b)** after 4 hours of detachment.

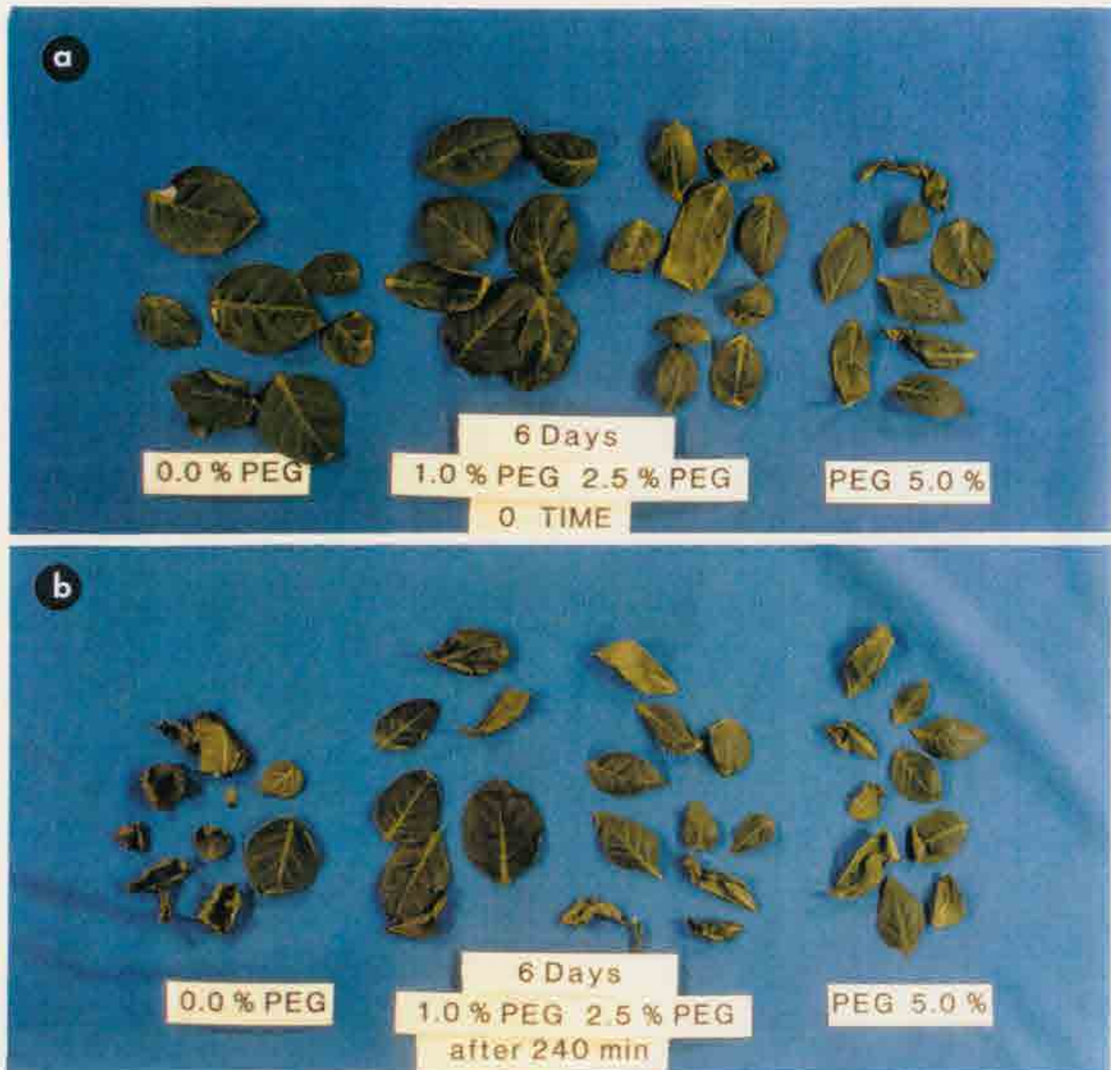

Figure 3.7. Detached leaves of in vitro tobacco plantlets grown with 0% (control), 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for 6 days **a)** at time of detachment and **b)** after 4 hours of detachment.

Figure 3.8. Detached leaves of in vitro tobacco plantlets grown with 0.0% (control), 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for 10 days **a)** at time of detachment and **b)** after 4 hours of detachment.

A concentration of 5.0% PEG was more injurious to the leaves than lower concentrations of 1% and 2.5%. Polyethylene glycol concentration of 1% caused some reduction in moisture loss, but induced little hardening as measured by moisture loss of detached leaves (Fig. 3.5). Concentrations of 2.5% caused significant reductions in moisture loss and caused some injury in leaves. Growth of new leaves was produced at all PEG concentrations but the size and number of new leaves decreased with increasing concentrations.

Wax content of leaves from plantlets treated with 0, 1.0, 2.5 and 5.0% PEG increased significantly with increasing PEG concentration and the duration of treatment (Table 3.9; Fig. 3.9). However, wax content of the greenhouse plantlets (physiological age similar to IVPs) was significantly lower ($P < 0.01$) than the control in vitro tobacco plantlets. Wax extracted from tobacco plantlets grown in "Plant Science Green House" (50-70% RH) and "PERC" greenhouses (RH 20-50%) were statistically similar.

Rate of water loss of detached leaves had a significantly negative correlation with leaf wax content as affected by PEG treatments ($r = -0.539, -0.611, -0.679$ for 3, 6, and 10 day-durations respectively; $p < 0.01$; Fig 3.10). However, since correlation tends to be underestimated when there is a curvilinear association, a polynomial regression analysis was conducted. The relationship between RML and wax content was curvilinear and was described well by a significant regression of RML on wax and wax^2 ($P < 0.01$). There were significantly different lines for each duration of treatment, the lowest line occurring for the 10 day-treatment (Fig. 3.10). This indicated that the effect of PEG concentrations on increasing wax and

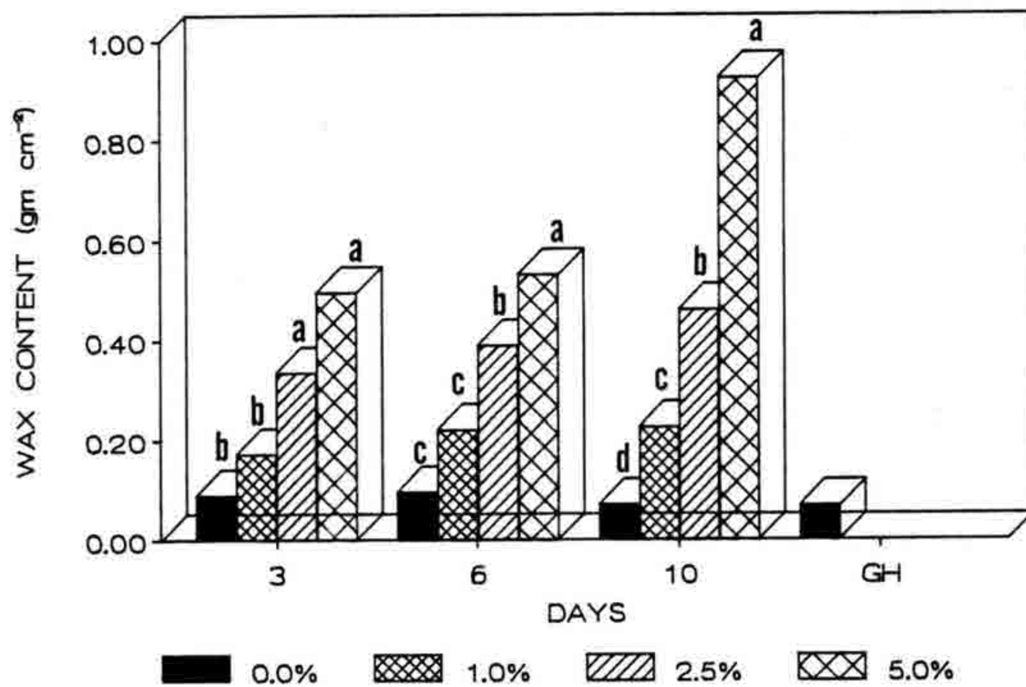

Figure 3.9. Leaf epicuticular wax content of in vitro tobacco plantlets treated with 0.0%, 1.0%, 2.5% and 5.0% polyethylene glycol (PEG) for 3, 6, and 10 days.

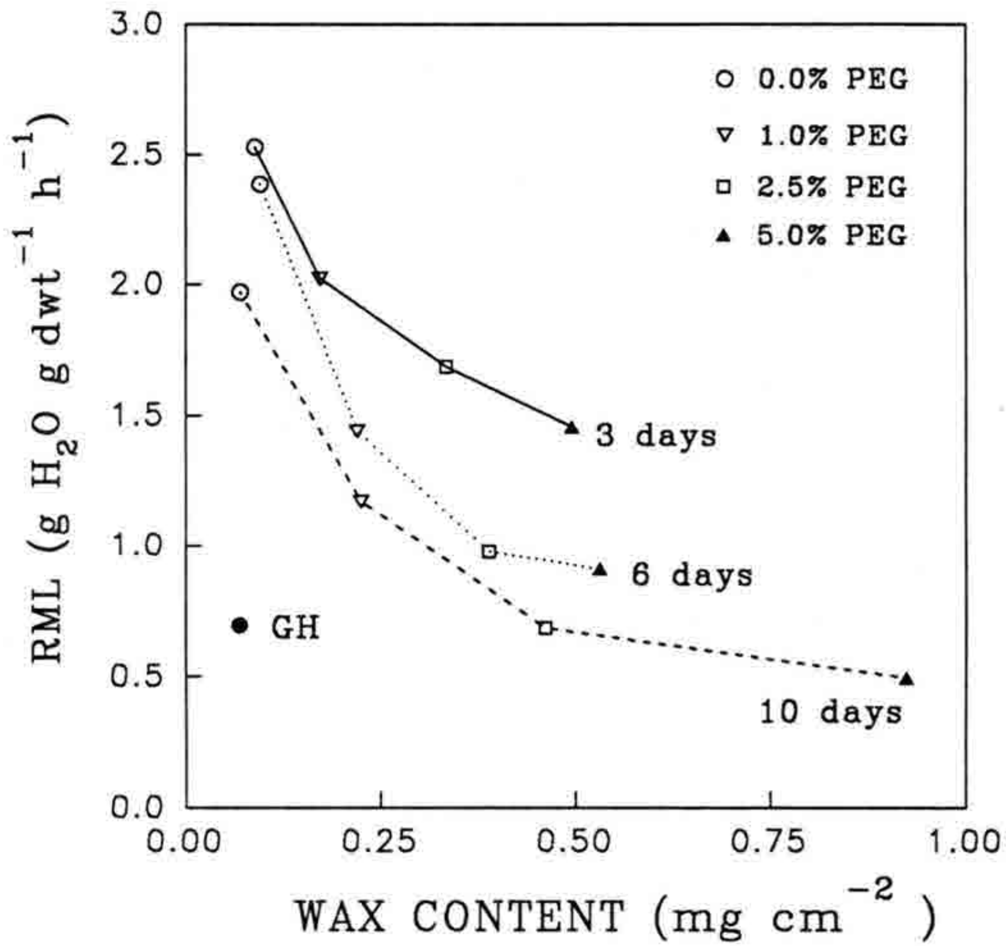

Figure 3.10. Relationship between wax content (mg cm^{-2}) and rate of moisture loss [RML: g H_2O g^{-1} dry weight (dwt) $hour^{-1}$ (h^{-1})] of plantlets treated with 0.0%, 1.0%, 2.5%, and 5.0% PEG and greenhouse (GH) plants.

reducing moisture loss was time-dependent with the longer treatment duration having a greater effect (R^2=0.46, 0.47, 0.6, for 3, 6, and 10 days respectively). However, wax content and RML of greenhouse leaves did not follow this relationship (Fig. 3.10; Table 3.9). The regression analysis using greenhouse as an indicator showed that rate of water loss was 1.08 units below the lowest line at the same wax level and that difference was significant ($P<0.01$; Fig 3.10).

b. Growth. PEG concentrations of 1.0, 2.5 and 5.0% reduced growth significantly as concentration increased (Table 3.10). The adverse effect of PEG on growth was greater when plantlets were incubated for a longer time with PEG (Fig. 3.11). The negative effect of PEG and length of treatment on growth was true for fresh weights, dry weights, leaf area and to a lesser extent number of leaves (Table 3.10). The constant number of leaves shows that the effect is on stem elongation, leaf growth and expansion. Root dry weight was not reduced by the PEG treatment. On the contrary, an increase in dry weight was observed (Table 3.10).

D. Discussion

The high rates of water loss observed in detached leaves from untreated tobacco plantlets are consistent with previous reports for leaves in other species (Brainerd and Fuchigami, 1981; Fuchigami *et al.*, 1981; Conner and Conner, 1984; Dhawan and Bhojwani, 1987; Zaid, 1990; Dami, 1991; Grout and Aston, 1977; Wardle *et al.*, 1979; 1983). In the previous reports, leaves lost about 50% of their water content within the first 30 to 60 minutes after detachment and exposure to ambient air. In comparison, tobacco leaves lost 50% of their water content in 2 to

Table 3.10. Effect of concentrations of PEG on growth of tobacco plantlets treated for 3, 6 and 10 days.

% PEG	Days	Fwt[1] (g)	Dwt[2] (g)	leaf area (cm^2)	stem length (cm)	No. of leaves	Root Dwt (g)
0.0	3	2.30 a[z]	0.15 a	82.6 a	4.0 a	11.0 a	--
1.0	3	1.70 b	0.13 b	61.4 b	3.8 a	10.6 a	0.04 a
2.5	3	1.50 b	0.12 b	56.3 b	3.6 a	10.6 a	0.04 a
5.0	3	1.20 c	0.12 b	43.1 c	2.7 b	10.1 a	0.05 a
0.0	6	1.80 a	0.13 a	75.9 a	2.8 a	11.3 a	0.05 a
1.0	6	1.30 b	0.12 ab	58.2 b	2.8 a	10.2 b	0.05 a
2.5	6	1.20 b	0.12 b	44.5 c	2.6 a	11.2 a	0.06 a
5.0	6	0.90 bc	0.10 b	37.6 c	2.4 a	10.1 b	0.06 a
0.0	10	1.99 a	0.16 a	82.9 a	4.3 a	10.9 a	0.05 a
1.0	10	1.28 b	0.14 b	52.8 b	3.9 a	10.8 a	0.05 a
2.5	10	0.91 c	0.12 bc	37.8 c	3.1 b	10.3 a	0.05 a
5.0	10	0.56 d	0.11 c	27.7 c	2.5 b	9.20 b	0.06 a

[z]Mean separation using Student Newman Keuls Test. Means followed by the same letter do not differ significantly at the .05 level of significance.

[1]FWT = Fresh Weight

[2]DWT = Dry Weight

Figure 3.11. Tobacco plantlets treated with 0.0%, 1.0%, 2.5% and 5.0% PEG showing the effect of PEG on growth of tobacco plantlets.

4 hours after detachment. Since a loss of 50% of the water content of the leaves of in vitro plums was found to correspond with cell injury (Kobayasi *et al.*, 1981), tobacco plantlets may be considered less vulnerable to transplanting shock than others. Indeed, tobacco plantlets were described to have transient in vitro features which were readily reversible upon transplanting to natural conditions (Pospisilova *et al.*, 1988). Variability in transplantability of plants have been reported (McKee 1981). Tobacco plantlets, however, show some signs of water stress injuries upon transfer to the greenhouse. Irreversible wilting of leaf edges or portions of the leaves takes place which later becomes necrotic (Safadi and Hughes, 1991).

Pre-conditioning plants to water stress by exposure to moderately low Ψ_ω medium has been used to improve water stress resistance or acclimation in some crop species (Kaul, 1966; Jensen, 1981; Janes, 1966; Seiler, 1985; Hasegawa *et al.*, 1984). Water stress pre-conditioning conferred water stress resistance features related to leaf size, leaf morphology, epicuticular wax content, stomatal conductance and osmotic adjustment (Seiler, 1985; Kramer, 1983; Clemens and Jones, 1978; Matthews and Boyer, 1984.

Decreasing the water status of tissue culture plantlets was suggested as a major method of acclimatization of plantlets (Ziv *et al.*, 1983, 1987; Ziv 1986; Wardle *et al.*, 1983; Short *et al.*, 1987; Debergh *et al.*, 1981). Pre-conditioning tissue culture plantlets to water stress using osmotic agents to reduce Ψ_ω of the rooting medium was thus attempted in this work.

The use of PEG as an osmoticum in liquid medium conferred some water stress resistance to in vitro plantlets as measured by the reduced water loss of the

leaves and increased wax content. Resistance was provided by the lower concentrations of PEG at 1.0%, 2.5%, and 5.0% and the shorter duration (3 days) of treatment with higher concentrations of 10%, 15%, and 20% PEG. The longer duration treatments with high concentrations of PEG increased water loss of the detached leaves, possibly due to leaf injury which was observed visually. Growth data also showed inhibition at the higher concentrations. Even the low concentrations of 2.5% and 5.0% reduced growth as compared to the control.

Explants started on solid medium supplemented with PEG exhibited inhibition of growth and rooting, possibly due to the severe water stress applied by the 1.0% and 1.5% PEG within agar. The Ψ_w of this medium at 1.0% and 1.5% PEG was equivalent to that exerted by 10% PEG in liquid medium, while 0.25% PEG resulted in Ψ_w equivalent to 5.0% PEG (Table 3.1).

These results are consistent with literature where reduction of Ψ_w of the tissue culture medium using increased agar concentrations of 1.4 to 1.5% overcame vitrification and produced normal glaucous plantlets with increased epicuticular wax formation, normal stomatal function, and improved survival in other plant species (Ziv *et al.*, 1983; Leshem, 1983; Wardle *et al.*, 1983; Debergh *et al.*, 1981; Bornman and Vogelmann, 1984; Marin and Gella, 1987; Singha *et al.*, 1990). Growth retardation of in vitro plantlets grown under high agar concentrations was ascribed to excessive water stress on the plantlets although earlier reports suggested an inhibitory effect of agar on cytokinin availability in the medium (Debergh *et al.*, 1981). According to these authors the beneficial effects of the decreased Ψ_w of the

medium on acclimatization and survival of plantlets is offset by its deleterious effect on growth.

Using PEG in the rooting medium of chrysanthemum increased epicuticular wax production and improved survival (Short *et al.*, 1987). Similar treatments increased epiculticular wax content and decreased water loss in date palms and grapes (Zaid, 1990; Dami, 1991) as well as induced more normal leaf anatomy in grapes (Dami and Hughes, 1991b). In these reports, severe growth retardation due to PEG occurred in both chrysanthemum and grape plantlets.

The effect of water stress on reducing growth is a well known phenomenon (Acevedo *et al.*, 1971; Hsiao and Bradford, 1983; Salisbury and Ross, 1985). This is largely due to the effect of reduced Ψ_w on metabolic processes involved in the expansive growth, photosynthesis and other metabolic processes (Hsiao et and Bradford, 1983; Salisbury and Ross, 1985; Jensen, 1981). Processes such as mineral uptake and stomatal responses (Kawasaki *et al.*, 1983; Jensen, 1981) are also affected.

Polyethylene glycol as an osmotic agent used to simulate water stress reduces Ψ_w (Janes, 1966; 1974; Kaufmann and Eckard, 1971; Jensen, 1981) and growth (Kawasaki *et al.*, 1983; Ciamporova and Luxova, 1975; Janes, 1966; Prasad *et al.*, 1982; Michel, 1970; Parmar and Moore, 1968; Jackson, 1962). The effect of PEG on growth and leaf injury was largely attributed to osmotic effect on plant (dehydration) due to its presumed impermeability into tissues (Janes, 1974; West *et al.*, 1980; Jensen 1981; Janes, 1966).

Although high mw PEG is considered not to penetrate plants, several reports have noted non-osmotic effects of PEG on growth such as toxicity associated with permeability (Janes, 1974; Lawlor, 1970; Kaufmann and Eckard, 1971; Yaniv and Werker, 1983). Non-osmotic effects also included presence of aluminum and magnesium metallic ion contaminants (Lagerwerff *et al.*, 1961) or organic impurities in PEG (Greenway *et al.*, 1968), inhibition of phosphorous uptake and transport across the root to the xylem (Resnik, 1970), and low solubility of oxygen in PEG-containing solutions (Mexal *et al.*, 1975). PEG also reduced mineral (Kawasaki *et al.*, 1983) and water uptake by plugging water pathway in the roots (Lawlor, 1970). However, PEG of high molecular weights (above 4000) are generally considered to be non-penetrating osmotica compared to low molecular weight PEG solutes, mannitol and ionic salts (Michel, 1970; 1971; Kaufmann and Eckard, 1971; Hasegawa *et al.*, 1984). Janes (1974) found that PEG accumulation in plants was inversely related to the molecular size and directly related to the time of exposure and the decrease in Ψ_{π} of the nutrient solution. However, Lawlor (1970) found that PEG 4000 entered freely into plants with broken or injured roots and accumulated in the leaves causing severe injury. Krizek (1985) accordingly, cautioned against the use of injured roots in water stress experiments involving PEG. Our method of treating plantlets with PEG 6000 included handling the plantlets by pulling them out of the agar medium and cleaning the agar off the fine hairy roots. Thus, it may be likely that the roots were broken during this process and PEG penetrated the broken roots causing toxic effects. Furthermore, roots of tissue culture plants have

delicate tissues which slough easily upon transplanting (McClelland *et al.*, 1990). These conditions may predispose plants to injury due to PEG penetration.

PEG was transported in some plant tissues without being broken down and was isolated and identified from leaf surfaces (Lagerwerff *et al.*, 1961; Lawlor, 1970; Janes, 1974). Yaniv and Werker (1983) observed absorption by roots and secretion of PEG 1500, 4000, and 6000, when used at 4.5% concentrations, by leaves of 8 Solanaceous plant species especially in mechanically damaged roots. These seedlings lost turgor, dropped their leaves and collapsed if kept on PEG-containing medium more than 4 days (Yaniv and Werker, 1983). A white deposit which was secreted through the non-glandular hairs of these plants was observed on leaf surfaces after they dried (Yaniv and Werker, 1983; Lagerwerff *et al.*, 1961). Since tobacco is a Solanaceous plant, it is possible that penetration of PEG took place causing injury of the tissues. In the process of chloroform-wax extraction from tobacco leaves treated with PEG, we observed a precipitate of white material at the edges of beakers used for treated plants, but not the control. This material was not identified; however, it was not soluble in chloroform but readily soluble in water. PEG-treated leaves were observed to be sticky, especially those that had been injured.

Different plant species may respond differently to PEG treatments. In our laboratory, date palm (Zaid, 1990) and strawberry plants were treated with 20% PEG with little injury while grape plantlets were treated with 10% PEG for 10 days without visible injury (unpublished results). Uptake of PEG, therefore, appears to be greatly dependent upon plant material and root condition (Krizek, 1985).

Three additional factors may have contributed to the non-osmotic effects of PEG on tobacco injury in culture. Firstly, the low oxygen solubility in culture solutions containing PEG generally necessitates shaking or aeration of the cultures (Mexal *et al.*,, 1975; Janes, 1966; Lawlor, 1970; Kaul, 1966). Secondly, the high viscosity of PEG caused gradients in concentration around the roots which rendered diffusion of nutrients into the roots more difficult (Kaul, 1966). In addition due to its low diffusability, PEG was found to concentrate in a boundary layer solutions around the roots thus inhibiting mineral and water uptake (Michel, 1971). To overcome this problem continuous automatic circulation of PEG mineral solutions is required (Kaul, 1966). Stirring of such solutions reduced adverse effect on cucumber hypocotyl elongation (Michel, 1971). Thirdly, plants adjust better to water stresses which occurred gradually in a manner similar those occurring in nature (Janes, 1966). Abrupt change of osmotic pressure of the solution from 0.5 to 10.0 bars caused osmotic shock, wilting and injury of plants (Janes, 1966; Krizek, 1985). When gradual reduction of Ψ_w of the medium was done by adding small amounts of PEG to reduce Ψ_w in steps of one bar daily, plants adjusted to stress without wilting or injury (Janes, 1966). In addition, PEG could interfere with normal water relations of plants. Plants grown under high humidity exhibited guttation when grown in PEG solutions resulting in sap containing PEG molecules (Kaufmann and Eckard, 1971; Michel, 1971).

All these factors may explain the reduced growth and injury of tobacco plantlets upon treatment with PEG. The beneficial effect of PEG that we obtained on reducing water loss of leaves could possibly be augmented if the treated liquid

medium cultures were aerated or shaken and stress applied gradually to non-broken roots. The solid medium supplemented with PEG may have had inconsistent concentrations due to precipitation of PEG during medium dispensing or cooling. This may explain the large variability in growth obtained in these experiments.

The basis for the PEG effect on reducing water loss is possibly due to stomatal closure and/or increased epicuticular wax content. The effect of PEG on reducing transpiration has been reported by several investigators where transpiration was reduced as a result of low Ψ_w on stomatal physiology (Janes, 1961; 1966; 1974; Lawlor, 1970; Davies *et al.*, 1981). Water stress pre-conditioning caused irreversibly reduced transpiration in Black Alder (Seiler, 1985).

In this study the leaves from treated plantlets had significantly reduced transpiration even immediately after transfer (0 time; Fig 3.5). The shape of the curve, however, does not show the bi-phasic shape of stomatal and cuticular water loss typical of normal responses of detached leaves to increased vapor pressure deficits (McCaig and Romagosa, 1989). In contrast, the shape of the curve for greenhouse leaves conforms to these responses. This shows that the stomates were closed before transfer and maintained high diffusion resistance due to the effect of low Ψ_w induced by PEG. Similar results were obtained by Seiler (1985) and Lawlor (1970). In another part of this study porometric measurements as well as microscopic observations of stomates from PEG-treated leaves revealed high diffusion resistances and closed stomates (Safadi *et al.*, 1990).

The lower initial transpiration of detached leaves of treated plants may also be due to the increased wax deposition that we have detected. Wax deposition was

also reported as a result of water stress in the growing medium (Sutter and Langhans, 1982; Ziv *et al.*, 1983; Seiler, 1985; Esau, 1977). However, greenhouse plants had negligible amounts of epicuticular wax yet a low rate of water loss. This indicates the minimal importance of waxes in water conservation. In addition, we did not detect a straight line relationship between wax content and RML. This is confirmed in the literature where tobacco plants were found to be "nonwaxy-bearing" plants (Scheiferstein and Loomis, 1956). It also agrees with the findings of Sutter (1985) who demonstrated inconsistencies in wax content and appearances among different in vitro and in vivo plant species. Sutter (1985) also determined that epicuticular wax of in vitro plantlets is not necessarily associated with rates of water loss and survival. Bengston *et al.* (1987) and Denna (1970) also failed to detect a straight line relationship between wax content and RML.

There has been a controversy about the role of epicuticular waxes in water conservation (Daly, 1964). Scheiferstein and Loomis (1956) and Hull *et al.* (1975) underscored the importance of epicuticular wax in xerophytic adaptations in favor of a continuous cuticular layer possibly embedded with intracuticular wax. Several others, however, have indicated the importance of epicuticular wax in adaptations to arid environments (Daly, 1964; Denna, 1970, Seiler, 1985).

The reduced leaf size of water stressed plantlets and some desert plants is known to be one of the xeromorphic adaptations of plants (Esau, 1977; Seiler, 1985; Dale, 1982; Salisbury and Ross, 1985). Thus reduced leaf size as induced by the PEG-treatments tobacco plantlets could be associated with reduced rates of water loss.

Leaves from PEG-treated plantlets could also have undergone osmotic adjustment. This osmotic adjustment may be implied by the increased dry weight to fresh weight ratios in the PEG treated plants (Table 3.5). Osmotic adjustment of PEG-treated plantlets by accumulation of organic solutes and to a less extent inorganic ions (Ca^{++}, Mg^{++} and K^{+}; if available) was reported by Jane (1966) and Jensen (1981) and was indicated by increased dry weight to fresh weight ratios. Osmotic adjustment was reported for tobacco plantlets grown on relatively low Ψ_{ω} medium solutions which subsequently disappear after transfer to soil solutions with higher Ψ_{ω} (Pospisilova *et al.*, 1988). Osmotic adjustment of in vitro plantlets is useful in stimulating better water uptake upon transfer (Pospisilova *et al.*, 1988).

Reducing Ψ_{ω} of the growing medium using ionic salts has also been used in water stress (Slatyer, 1961; Janes, 1966; Kawasaki *et al.*, 1983; Jensen, 1981; Parmar and Moore, 1968) and in acclimatization (Heyser and Nabors, 1981; Binzel *et al.*, 1986) studies. In this experiment salt treatments with equal amounts of NaCl and $CaCl_2$ reduced water loss of detached leaves from in vitro plantlets. However, reduction of water loss was greater with salts at the higher concentrations of 1.5% and 2.0% and the longer duration of treatments (6 and 10 days).

Although wax content and stomatal apertures were not measured, reduction in moisture loss might be attributed to either one. The effect of salts on cuticular wax formation and stomatal anatomy was previously reported by McNeilly *et al.* (1987). They observed increased leaf waxiness, epidermal hair and sunken stomates in plants grown in salt medium. Salt exposures also reduced rates of transpiration as long as the roots were in low Ψ_{π} (Janes, 1966; 1974; Seiler, 1985).

Similar to PEG-treated plantlets, the reduced water loss in tobacco plantlets treated with 1.5%, and 2.0% salt for 6 and 10 days was observed immediately after transfer. However, the shape of the curve of water loss of leaves from salt-treated plantlets as compared to that of the greenhouse did not follow the typical bi-phasal response of stomates of detached leaves to increased vapor pressure deficits. The water loss curves in salt treated plants indicated a reduction in transpiration which occurred due to osmotic effects of the salt solution. This adaptation could be advantageous in reducing rates of water loss at the time of transfer until the plantlets restore their water balance but does not relate to the normal responses of plants to ex vitro environmental conditions.

In spite of the advantageous effect of salts in reducing water loss, plants treated with salt show peculiar growth. These plants appeared succulent with significantly higher water content and fresh weights with increasing salt concentrations (Table 3.9, 3.10). The higher dry weights as well as fresh weights of leaves without significant increases in the stem length or leaf number indicated that salts induced leaf expansion. This was visually observed in the salt treatments.

In practice, large leaves are not desirable for IVPs since they lead to more water loss upon transfer ex vitro, which is in turn conducive to the development of water stress (Desjardins *et al.*, 1987). In addition, succulence in IVPs is one of the major problems of cultured plantlets predisposing them to vitrification (Ziv *et al.*, 1983). The salt treatments utilized in these experiments therefore, based upon their growth characteristics, may not be considered useful osmotica in hardening of IVPs. In addition, the higher concentration treatments which conferred reduction in

moisture loss resulted in leaf chlorosis with signs of mineral deficiency and subsequent necrosis.

These above results are consistent with the findings of Flowers *et al.* (1986) who noted increased succulence of tobacco seedlings with increasing salt concentrations. They observed increased leaf water content and fresh weights but not dry weights or leaf number. Succulence is known to be a common response of plants to salts (Salisbury and Ross, 1985; Esau, 1977). In our results we observed increases in dry weights as well as fresh weights over those of control plantlets. Succulence due to NaCl has been reported to be due to an increase in cell size and/or cell number (Flowers *et al.*, 1986). Our results indicate an increase in cell number as well as size due to the insignificant differences in dry weight to fresh weight ratios.

Our results of increased fresh weights and dry weights are in contrast to studies which reported reduced growth of plants upon exposure to NaCl treatments (Janes, 1966; Parmar and Moore, 1968; Kawasaki *et al.*, 1983). NaCl was found to reduce Ψ_w and its components as well as dry weights (Janes, 1966). Kawasaki *et al.* (1983) reported reduced growth of maize exposed to NaCl as well as inhibition of uptake of K, Ca, and Mg. The effect of NaCl on inhibiting absorption of K, Fe, Mg or N has been noted in several other studies reviewed by Greenway and Munns (1980). This is likely the cause of the observed chlorosis in our treatments with salt.

High Na concentrations in cells caused imbalances in Na^+/K^+ and Na^+/Ca^{++} ratios which affected membrane permeability (Kingsbury and Epstein,

1986; Cramer *et al.*, 1987). Calcium ion is known to counteract the toxic effect of NaCl by improving K^+ ion absorption and selectivity in membranes (Cramer *et al.*, 1987). A high Na^+:Ca^{++} ratio decreased growth and increased succulence in tobacco plantlets while a low ratio increased growth (Flowers *et al.*, 1986). High Cl^- concentrations were also found to be linearly related to succulence (Flowers *et al.*, 1986). In our NaCl:CaCl concentrations studied, the Na^+:Ca^{++} ratios must have been imbalanced to the point which caused succulence. The Cl^-ion concentration must have been high enough to cause succulence since it is the anion applied with Na^+ and Ca^{++}.

These studies have explored the possibility of using PEG and salt osmotica in acclimatizing in vitro plantlets. PEG at high concentrations, above 10% was injurious to tobacco plantlets. PEG added to solid medium caused greater reductions in Ψ_w than when added to liquid medium, thus resulting in inhibition of rooting and shoot growth. PEG concentrations of 1.0%, 2.5% and 5.0% and salt treatments of 1.5% and 2.0% were found to reduce the rate of water loss of detached leaves. Both osmotica caused some degree of injury to tobacco plantlets. PEG treatment reduced growth of plantlets while salt promoted growth but in an abnormal manner. The use of salts was more discouraging because of chlorosis and the succulent growth characteristics. Results with PEG were encouraging in terms of growth at the lower PEG concentrations.

A concentration of 2.5% PEG for 10 days was considered to be a reasonable water stress preconditioning treatment to induce water loss reduction in tobacco plantlets. However, the method of application of these treatments needs further

refinement. This may include aeration or circulation of PEG solution, avoiding root injury and gradual application of the stress treatment. The water relations of cultures treated with PEG should be carefully studied, since the PEG treatments could affect the medium-plant-atmosphere water transport continuum. The urgent need to study water relations of plantlets in culture has been repeatedly recommended by Kozai and his coworkers (Kozai and Iwanami, 1988; Kozai, 1988; Kozai, 1991). This indicates the necessity of a more thorough investigation relative to the use of osmotica in acclimatizing in vitro plantlets.

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CHAPTER IV

COMPARISON OF DIFFUSIVE RESISTANCE OF POLYETHYLENE GLYCOL-TREATED AND NON-TREATED IN VITRO TOBACCO PLANTLETS

Introduction

The low survival of in vitro produced plantlets (IVP) upon transfer to ex vitro conditions has been attributed to excessive water loss after removal from culture. Reduced epicuticular wax formation (Grout 1975, Sutter and Langhans 1979), non-functioning stomates (Wardle *et al.* 1979, Brainerd and Fuchigami, 1982, Wardle and Short 1983), insufficient water transport through abnormal root-shoot-xylem connections (Grout and Aston 1977) and lack of autotrophy (Grout and Aston 1978, Fujiwara *et al.* 1987) have all been reported as possible causes for the rapid water loss.

Brainerd and Fuchigami (1981) and Wardle *et al.* (1979) showed that lack of stomatal closure in IVP is the major cause for the desiccation and death of IVP upon transfer to ex vitro conditions. Stomates of IVP failed to respond to closure stimuli such as mannitol, ABA, darkness, CO_2, and Ca^{++} (Brainerd and Fuchigami 1982, Wardle and Short 1983, Ziv *et al.* 1987). Although earlier reports related water loss and low survival rates of IVP to lack of epicuticular wax coverage (Grout and Aston 1977, Sutter and Langhans 1979), the significance of the role of

epicuticular wax in the desiccation response of IVP has become increasingly questionable (Sutter, 1985, 1988). Stomatal malfunctioning and the lack of wax deposition were found to exist concurrently in IVP; the relative contribution of either one to water loss during acclimatization is the important question to be answered in correcting for abnormalities before transfer of plantlets from in vitro conditions (Sutter 1988).

In studies with apple *(Malus domestica* Borkh*)*, cherry (*Prunus avium xpseudocerasus*) and sweet gum *(Liquidambar styraciflua* L.) leaves, Sutter (1988) obtained closure of the stomates in response to reduced relative humidity during an acclimatization treatment. Cuticular conductance (typical of the extent of epicuticular wax) was variable and was confounded by desiccation (Sutter 1988). Shackel *et al.* (1990) obtained incomplete stomatal closure of in vitro apple plantlets after an acclimatization treatment of 90% RH. Cuticular conductances of in vitro apple plantlets were similar to those observed for field-grown apple plants. His study suggested that lack of acclimation for IVP was due to nonfunctional stomates, but also that conditioning at 90% RH was insufficient for acclimating plants prior to transplanting.

Acclimatization of IVP before transfer from culture is recommended to induce a more stress-responsive physiology (Grout and Aston 1977, Brainerd and Fuchigami 1981, Sutter 1988, Shackel *et al.*, 1990). Acclimatization methods that restore stomatal closure and improve survival include 1) reducing ambient humidity in vitro by uncapping the culture vessels (Brainerd and Fuchigami 1981), using a desiccant such as silica gel or $CaSO_4$ in the culture environment (Wardle *et al.*,

1983, Ziv *et al.*, 1987), or bottom cooling (Capellades *et al.*, 1990) and 2) reducing media water potential by increasing agar concentrations or PEG (Ziv *et al.*, 1987, Short *et al.* 1987).

Stomates are known to respond to changes in ambient humidity, the response being mediated through epidermal cell turgor (Davies *et al.* 1981, Schulze *et al.* 1987). Low soil water status can close stomates, even during exposure to light and low VPD, since hydraulic considerations override the photoactive and VPD responses of guard cells (Davies *et al.* 1981, Schulze *et al.* 1987). This phenomenon of rhizospheric-induced stomatal closure is utilized in the present study with in vitro cultured plantlets by adding the osmoticum, PEG to the culture medium in stage III. Previous studies indicated that this treatment reduces moisture loss of detached leaves from in vitro tobacco plantlets and increases wax content (Chapter III).

The main objective of this research was to study the acclimatization effect of PEG on stomatal response of in vitro tobacco plantlets. This was accomplished by monitoring leaf diffusive resistance (r) of in vitro and PEG-treated tobacco plantlets in two experiments: 1) detached leaves allowed to desiccate under laboratory conditions, 2) leaves on intact plantlets acclimatized by gradual reduction of humidity to avoid the confounding effect of desiccation on porometric measurements. This acclimatization to humidity allowed for an assessment of the plantlets' r response to increasing VPD and permitted a separation of acclimation responses into stomatal and cuticular effects.

Materials and Methods

1. Plant culture

Tobacco (*Nicotiana tabaccum* L. 'Wisconsin 38') plantlets were grown in vitro from single nodal sections cultured on 0.6% agar solidified Murashige and Skoog medium (Murashige and Skoog, 1962), with 3% sucrose and no hormones in 200 ml baby food jars. Rooted plantlets with 7 to 8 leaves were transferred to the same medium in liquid form, supplemented with 2.5% PEG (w/v). The plantlets were supported by filter paper bridges and grown for 10 days. In previous experiments, a 2.5% PEG and 10-day-period allowed for new growth under the acclimatization treatment and caused reduction of water loss of detached leaves. Growth room conditions were 100 μmoles m^{-2} s^{-1} photosynthetic photon flux density (PPFD) of continuous light at 25 $\pm$ 2°C, and 30-40% RH.

2. Gas exchange studies

a. Experiment 1. Diffusive resistance of detached leaves.

Plantlets treated with 2.5% PEG for 10 days were used. Leaf resistance of abaxial and adaxial surfaces of the most recently expanded leaves was measured with a steady state porometer (LiCor 1600, Lambda Inst. Corp. Lincoln, NE)[1]. The leaf was detached and r measured at 0, 30, 60, 120, and 240 minutes of drying under laboratory conditions of 30% RH, 23 $\pm$ 2°C, with approximately 10 μmole m^{-2} s^{-1} PPFD. The humidity in the cuvette was set at ambient RH. Total leaf resistance

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[1] Mention of a trademark or manufacturer by CSU or USDA does not imply its approval to the exclusion of other products or manufacturer that may also be suitable.

(r_l) was calculated according to Turner (1970) as

$$1/r_l = 1/r_{ab} + 1/r_{ad},$$

where r_{ab} and r_{ad} are the abaxial and adaxial resistances.

b. Experiment 2. Leaf diffusive resistance of acclimatizing intact plantlets

In vitro tobacco plantlets (7 to 8 leaf stage) were treated with 2.5% PEG for 10 days. Greenhouse-grown plants were also monitored for r. Greenhouse plants were started from seeds germinated in clay pots on peat 2 : vermiculite 1 : perlite 1 (by volume) in a growth chamber (Perceival, Boone, Iowa) at 25°C and 8/16 dark/light photoperiod. The seedlings were then transferred to the greenhouse and fertilized with Peters 20N-10P-20K. Temperature and RH in the greenhouse ranged between 20-27°C and 20% to 90% respectively. Greenhouse plants used for the measurements were of the same physiological stage of IVPs. Intact plantlets were removed from the culture vessel using a humidified chamber (100% RH) located in an EGC (M-11 Walk-in series, Environmental Growth Chambers, Chagrin Falls, Ohio) growth chamber. The roots were washed and immersed in water and plantlets were allowed to acclimate as follows:

1. Four hours in 85-90% RH and 280 μmole m^{-2} s^{-1} PPFD.
2. 20 hours in 70% RH and dark.
3. 24 hours in 30% RH and dark.

The dark treatments should induce stomatal closure and enable comparison of leaf resistances between PEG-treated and control plants, under conditions in which the potential effects of cuticular resistance should be maximal. The first fully expanded leaf from the apex was measured for r. Leaf resistance was measured at

0, 15, 30, 60, 120, and 240 minutes, and then at 24, 36, and 48 hrs after removal from culture and during the acclimatizing treatments. The temperature was maintained at 21 ± 2°C.

Data Analysis. In both experiments, four to six replicates (plants) of each treatment were used with the same leaf measured repeatedly at the indicated times above. The data were analyzed by SAS (1985) using analysis of variance for repeated measurements and Student-Neuman-Keuls test for mean separation at the 5% level of significance.

Microscopic observations. Stomatal closure was observed microscopically to relate stomatal closure with the porometric r data. Leaves comparable to those measured for r (first fully expanded leaf from the apex) were sampled for in vitro control, PEG-treated and greenhouse plants immediately after tramsfer, after 30 minutes of transfer and after 48 hours of acclimatization. Epidermal impressions of adaxial and abaxial surfaces were prepared by applying a thin pellicle of transparent fingernail polish. This was allowed to dry for 5 minutes and subsequently was peeled off with adhesive tape and mounted on a microscope slide.

Results and discussion

I. Experiment 1. Diffusive resistance of detached leaves.

Leaf resistance of PEG-treated leaves was about 3 times higher than that of the control at the time of removal from culture (Fig. 4.1). After 30 minutes leaf resistance increased about 6-fold in control leaves and 4-fold in PEG-treated leaves. This increase in resistance in the first 30 minutes could be either due to drying of

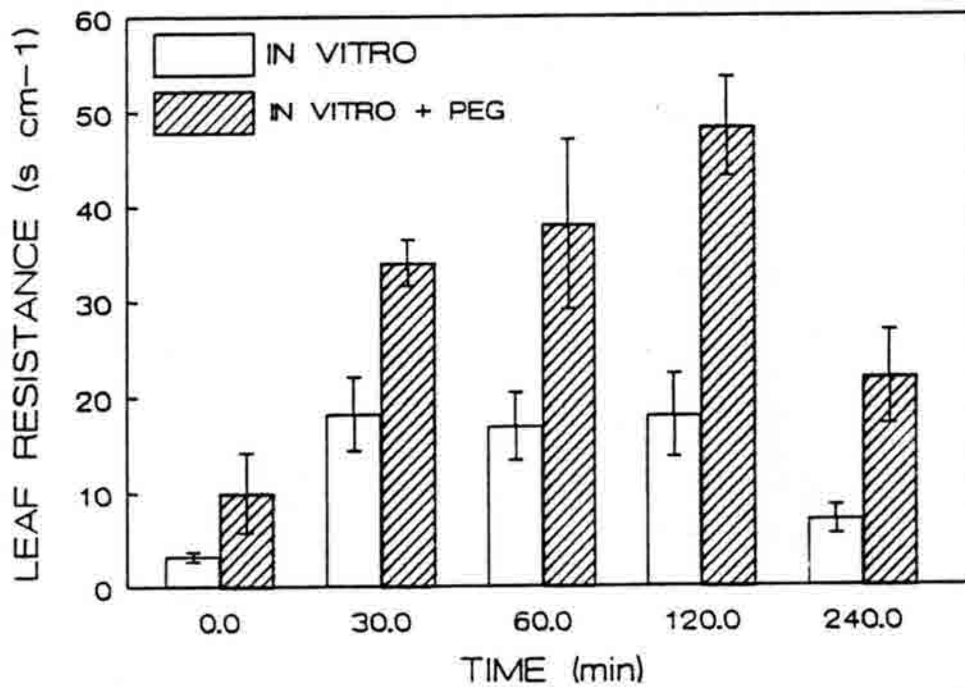

Fig. 4.1. Leaf resistance over time (min) for detached leaves of in vitro and PEG-treated in vitro tobacco plantlets exposed to 30% RH, 23°C and 10 μmole m^{-2} s^{-1}. Vertical bars represent $\pm$ standard error.

the moist leaf surfaces (from condensations in the in vitro jar), rapid stomatal closure induced directly by exposure to the dry conditions, or indirectly in response to the loss of tissue-derived water and shoot desiccation. The first and third possibilities were reported to explain some of the apparent decreased conductance of detached apple shoots (Shackel *et a*l. 1990).

The stomatal imprints (Fig. 4.2) obtained after 30 minutes support the desiccation possibility, since they show epidermal desiccation (epidermal cell shrinkage) and stomatal closure. The reduction in r after 240 minutes may be due to cellular damage caused by either severe desiccation or injury due to the porometer clamp. Similar results were obtained by Sutter (1988). In response to drying time, control plantlets' r remained more or less constant with time while that for PEG-treated plantlets increased. This indicates that PEG treatment may have induced more efficient stomatal functioning in response to increased VPD.

The significant finding is that PEG-treated leaves maintained higher leaf resistance. This may explain our studies in which reduced moisture loss was achieved in PEG-treated leaves (Chapter III). The results also support the notion of soil drought-induced stomatal closure (Davies *et al.* 1981, Schulze *et al.* 1987). However, the measured r is a measure of stomatal and cuticular resistances. The distinction between the two is necessary to elucidate the reasons of excessive water loss of IVP upon transfer ex vitro, and the means of acclimatization. The desiccating conditions of this first study did not permit evaluation of the conditioning aspects of stomatal closure (long-term acclimation response) separate from a stress response (short-term, rapid desiccation) (Sutter 1988, Shackel *et al.*

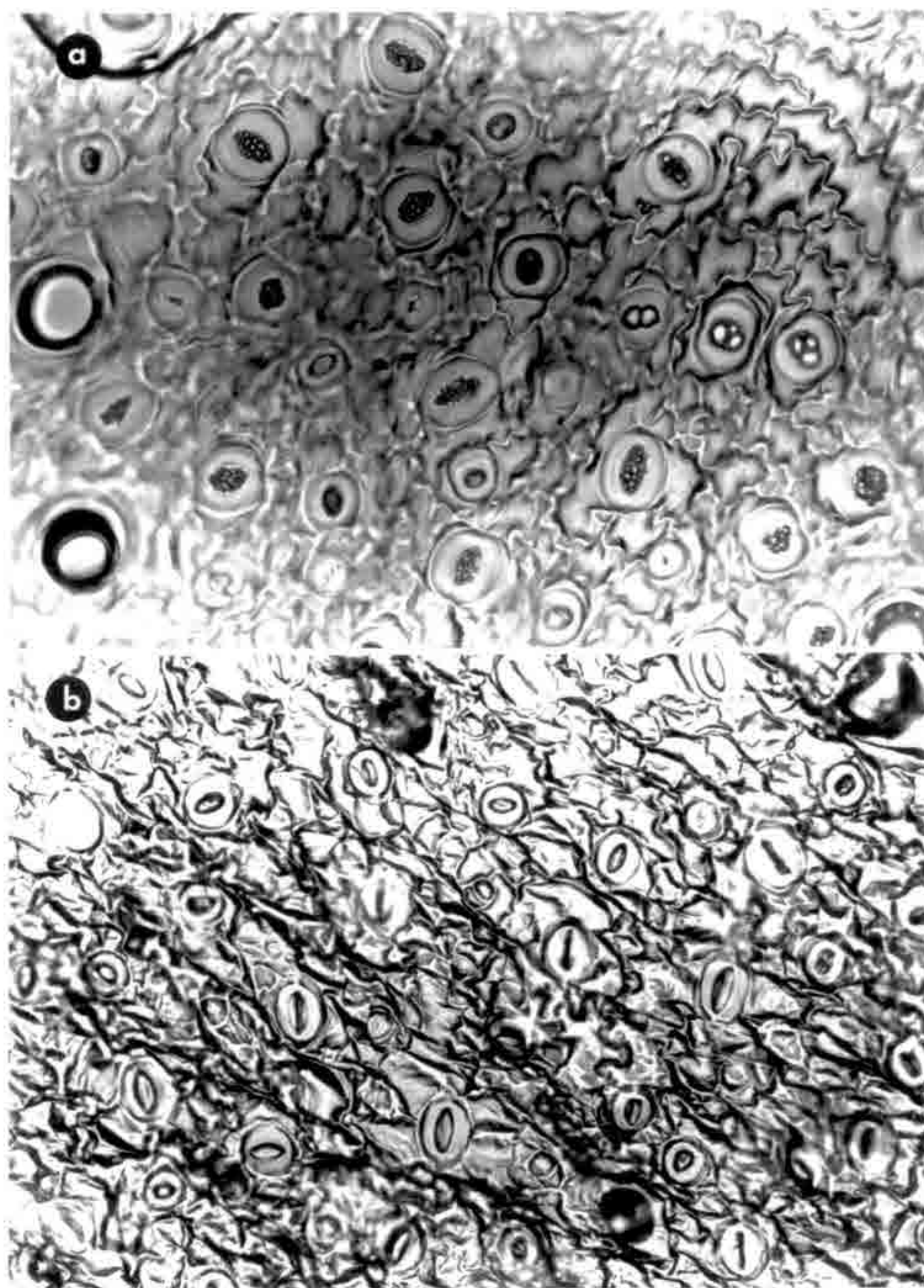

Fig. 4.2. Micrographs of leaf impressions showing abaxial stomatal apertures and epidermal cell size of non-treated IVP **a**) at the time of removal from culture and **b**) after 30 minutes following detachment showing desiccation of the epidermal cells (X250).

1990). Hence, a second study was undertaken to study the relative contribution of stomatal and cuticular resistances under conditions of lower VPD.

II. **<u>Experiment 2</u>. leaf diffusive resistance of acclimatizing intact plantlets.**

PEG-treated leaves showed an approximately 3-fold increase in r over control and greenhouse plants at time zero and consistently thereafter until 4 hours (Fig. 4.3). The extent to which these differences are due to increased stomatal closure and cuticular resistance cannot be elucidated until complete closure is achieved in all three treatments. Although there is a slight trend of increasing r in all treatments during the first 4 hours at high humidity and light, r did not increase significantly until leaves had been exposed to the dark-30% RH conditions. Dark treatment alone with 70% RH did not induce significant stomatal closure in the IVP, although it somewhat increased r in the greenhouse plants. It is reported that tobacco stomates normally close within 90 minutes in response to dark treatments (Zelitch 1961). In this experiment, the culture conditions of continuous light may have affected stomatal response to darkness. Imposition of photo-dark periods during culture conditions may be essential for better stomatal functioning.

Leaf resistance in Figure 4.3 indicates that stomates of IVP (control) seem to respond only to increased VPD applied after 36 and 48 hours. This may indicate partial stomatal functioning in response to VPD. However, the increase in r in the control plants lagged behind that of the PEG-treated and greenhouse plants, indicating the superiority in stomatal functioning of greenhouse over PEG-treated and PEG-treated over control.

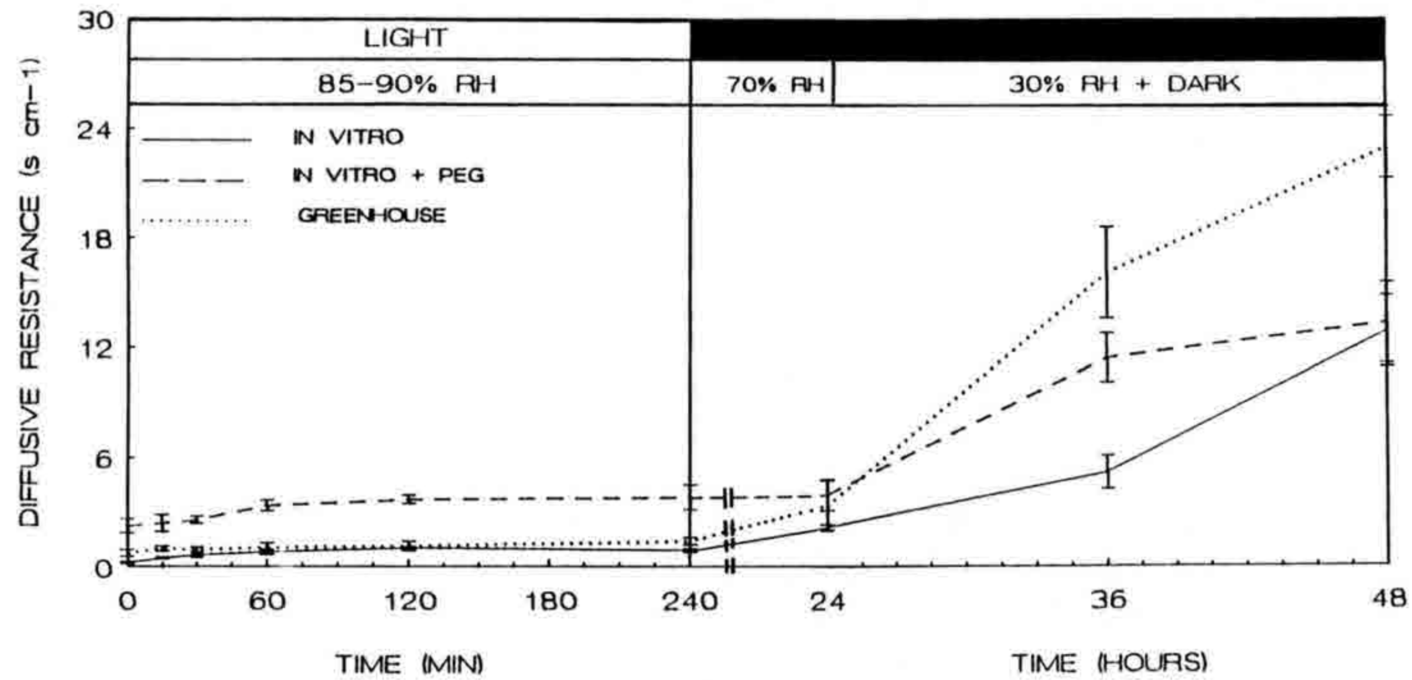

Fig. 4.3. Leaf resistance of intact in vitro and PEG-treated plantlets over time in comparison with greenhouse-grown plants. Vertical bars represent ± standard error.

Stomatal imprints made immediately after removal of plantlets from culture (0 min) show wide-open stomates for control leaves of IVP as compared to those from PEG-treated plantlets (Fig. 4.4 a,b,c,and d). This corresponds with the measurements of r (Fig. 4.3). After 48 hours, insignificant differences in r between PEG-treated and control IVP were observed (Fig. 4.3). Greenhouse plants had significantly higher r than both PEG-treated and control IVPs. Microscopic indications of stomatal apertures after 48 hours show incomplete stomatal closure of IVPs (Fig. 4.5 a, b, c, and d) and more complete stomatal closure of green house plants (Fig. 4.6 a, b). This again corresponds with the r data obtained using the steady state porometer. Shackel *et al.* (1990) also reported incomplete stomatal closure of IVP at the end of the acclimatization period.

The similarity in r between control and PEG-treated IVP after 48 hours suggests similar cuticular r for both treated and non-treated plantlets. Thus the higher r in the treated leaves at 36 h appears to be a stomatal closure response, induced by the low water status of the media. These results are consistent with observations of Shackel *et al.* (1990) in which in vitro apple cuticular conductances were within the range commonly found in field-grown plants. Previous reports attributing the high transpiration in IVP to stomatal malfunction suggest variation in epicuticular wax coverage may be less important in controlling water loss from IVP (Brainerd and Fuchigami, 1981; Sutter 1988, 1985).

The apparent trend of increasing r in both the control and the PEG-treated plants during the first 240 minutes may show that the plant is undergoing some degree of water stress, and that water transport lags in replenishing leaf water loss.

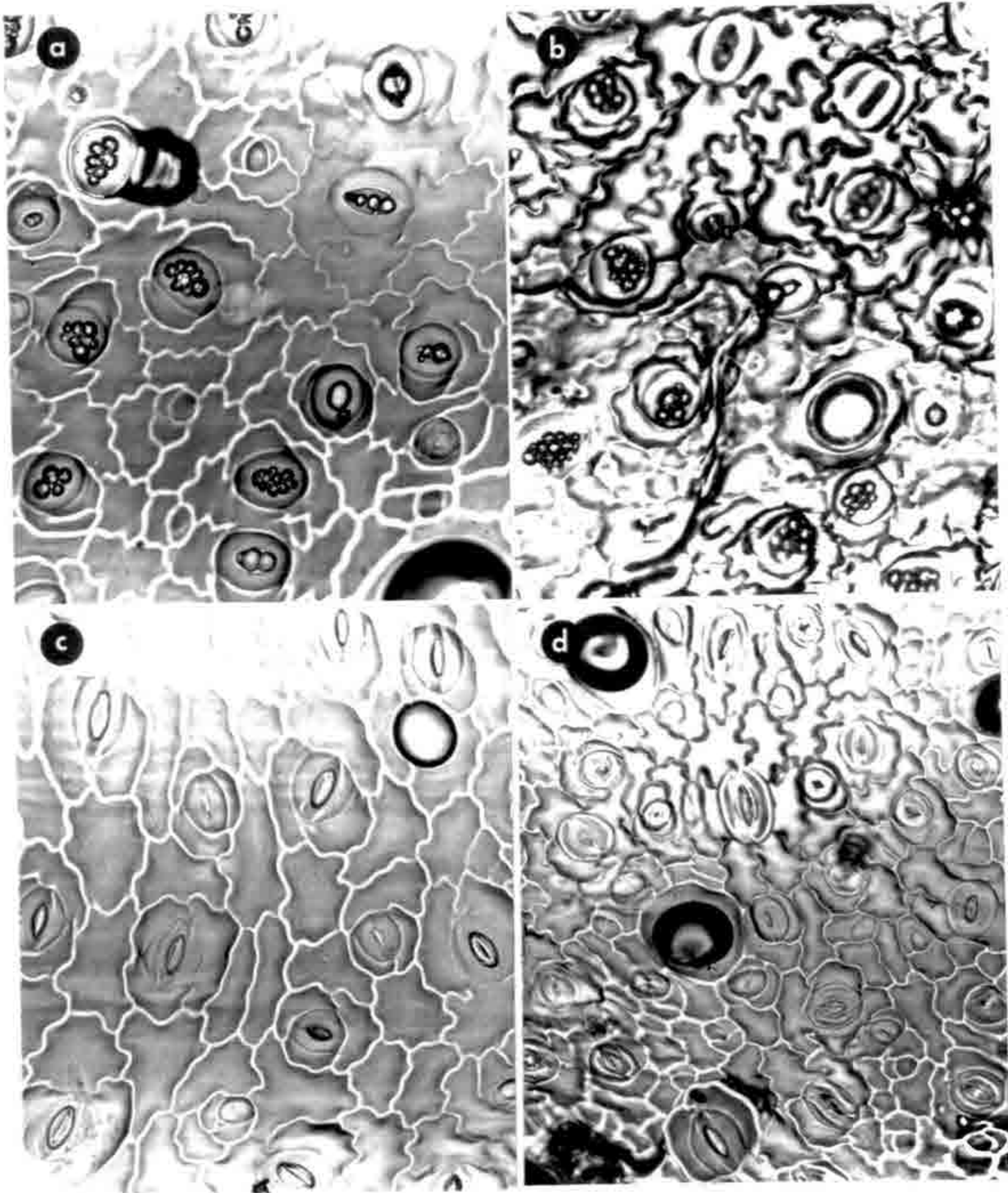

Fig. 4.4. Micrographs of leaf impressions of **a**) adaxial **b**) abaxial surfaces of IVP leaves and **c**) adaxial **d**) abaxial leaves of PEG- treated IVP immediately after removing from culture vessel (time zero) (X250).

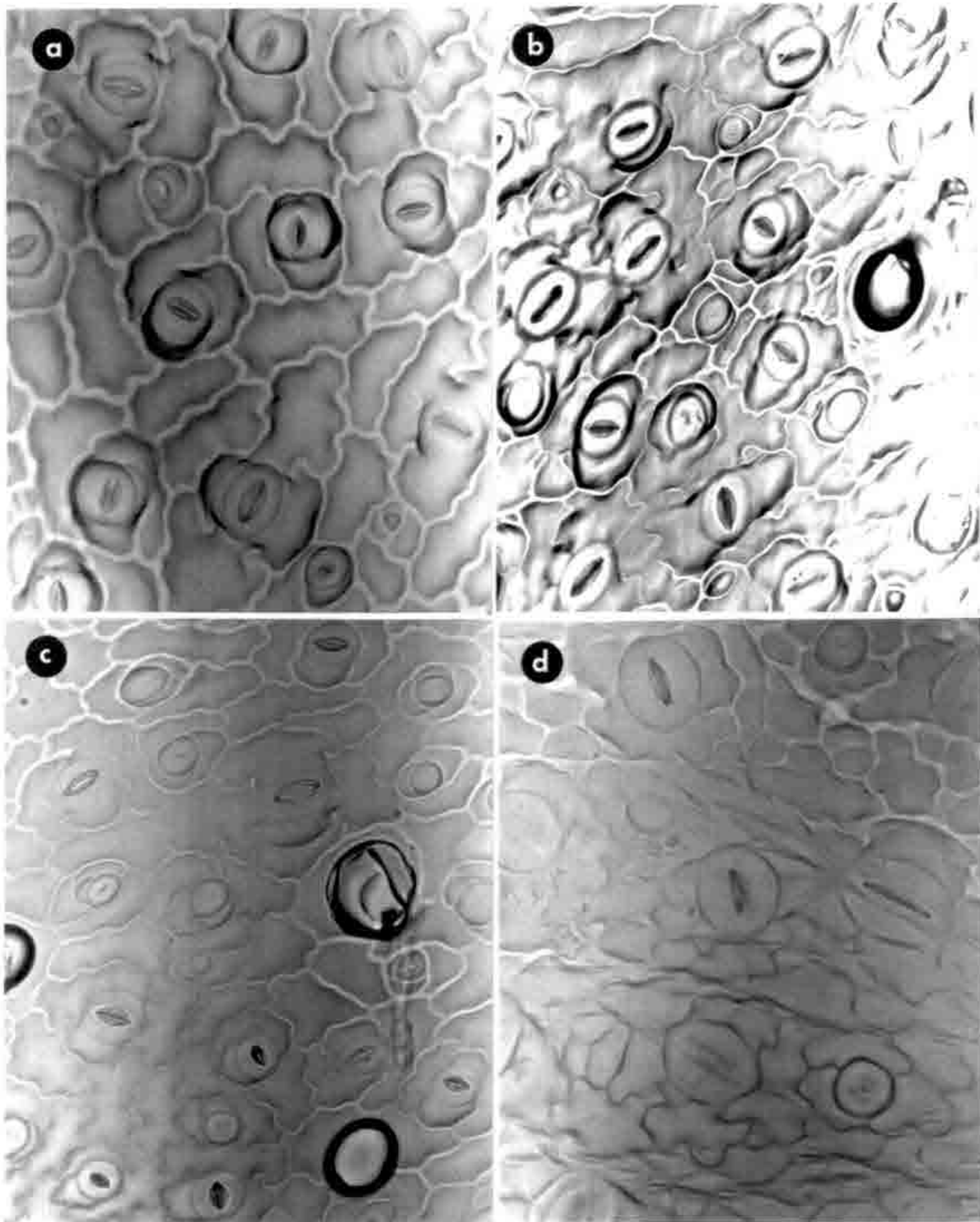

Fig. 4.5. Micrographs of leaf impressions **a**) adaxial and **b**) abaxial surfaces of IVP leaves (X250), as well as **c**) adaxial (X250) and **d**) abaxial surfaces (X500) of PEG-treated plantlets after 48 hrs of acclimatization.

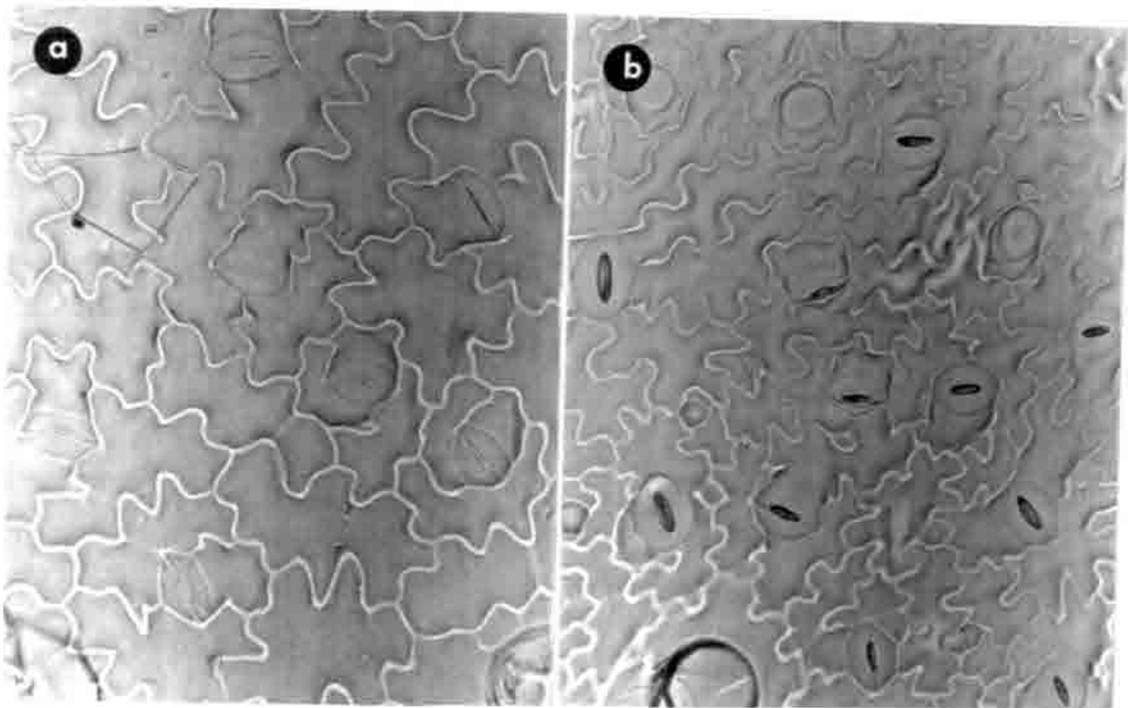

Fig. 4.6. Micrographs of leaf impressions of **a**) adaxial and **b**) abaxial surfaces of greenhouse plants after 48 hours of acclimatization (X250).

Roots were well developed and were not affected by the PEG (Chapter III). This finding is consistent with Shackel *et al.* (1990) who reported decreased leaf water content in acclimating apples under 90% RH. It is also known that plants at low VPD (as in the conditions of in vitro growth) have low water fluxes (Schulze *et al.* 1987).

Thus, an acclimatization period is necessary to restore stomatal closure and establish normal water fluxes in the xylem. A study of the water transport in IVP may be necessary to further investigate this point.

In conclusion, stomatal closure occurred with PEG treatment in vitro before removal of the IVP from culture. This significantly reduced transpiration and wilting in the first critical hours after transfer (Fig. 4.7). PEG treatment improved the effectiveness of stomatal response to increased VPD. The effectiveness of this method in acclimatization remains to be tested by monitoring survival under greenhouse conditions. The increase in r induced by the PEG treatment is primarily due to a decrease in stomatal aperture rather than an increased cuticular resistance. Desiccation of plantlets occurred after one hour (Fig. 4.2 and 4.7) at 30% RH, and some evidence of water stress occurred during the first 4 hours in spite of high RH of 85%. Further studies are needed to test the efficiency of the root system for water transport to replace the water lost through transpiration.

Fig. 4.7. Photographs of **a**) IVP and PEG-treated plantlets at zero time **b**) desiccating control tobacco plantlet after one hour ex vitro under the laboratory conditions, compared to PEG-treated plantlets, and **c**) tobacco plantlets after 48 hours of acclimatization.

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CHAPTER V

SEMIPERMEABLE COVERS ENHANCE ACCLIMATIZATION OF MICROPROPAGED TOBACCO PLANTLETS

Introduction

High water status of plantlets in tissue culture and their low photosynthetic rates are major factors causing poor acclimatization of plantlets upon transfer to ex vitro conditions. The high humidity (near 100% RH) under which plantlets in culture grow disrupts their medium-plant-atmosphere water transport continuum (Jarvis and Morison, 1981; Kozai, 1988; Maene and Debergh, 1987; Ziv, 1986). This promotes water retention, reduced mineral uptake and an abnormal physiology of in vitro plantlets (IVP; Kozai, 1988).

Poor development of epicuticular wax, stomatal malfunction and abnormal leaf structure and vascular system are features of IVP associated with high transpiration rates and low survival of plantlets transferred to greenhouse conditions (Sutter and Langhans, 1979; Brainerd and Fuchigami, 1981; Grout and Aston 1977; Wardle *et al.*, 1979; Donnelly and Vidaver, 1984). Much of the research on methods to facilitate acclimatization of IVPs has been directed towards modifying the water status of the tissue culture vessel and plantlets. Decreasing the matric potential of the medium by raising agar concentrations improved epicuticular wax development, induced stomatal function and caused glaucousness in carnation

(*Dianthus caryophyllus* 'Ceris Royallete'; Ziv *et al.*, 1983), and increased wax content in cauliflower (*Brassica oleracea* L. 'Botrytis'; Short *et al.*, 1987). Reduced medium water potential (Ψ_w) by adding polyethylene glycol (PEG) to the rooting medium reduced stomatal aperture in tobacco (*Nicotiana tabaccum* 'Wisconsin 38'; Safadi *et al.*, 1990) plantlets. Increasing agar concentration in culture medium improved survival rates and eliminated vitrification (in vitro characteristic of plants showing succulence, translucency and abnormal leaf morphology) in many plant species (Debergh *et al.*, 1981; Debergh, 1983; Bornman and Vogelmann, 1985; Leshem, 1983). These methods, however, reduced shoot multiplication and growth substantially unless they were applied after growth had been established (Ziv *et al.*, 1983).

In other studies, improved acclimatization through reduction in RH of the tissue culture environment has been successful. Brainard and Fuchigami (1981) enhanced acclimatization, reduced water loss and restored stomatal function of apple, *Malus domestica* (Borkh) 'Mac9', by uncapping cultures in the last 4-5 days before transfer. Lanolin overlays on the medium reduced RH and plantlet water loss but were detrimental to shoot growth (Wardle *et al.*, 1983; Crane and Hughes, 1990). Uncapping culture tubes in a desiccator or including a desiccant such as silica gel in the culture vessel induced normal leaf development and improved acclimatization but reduced growth depending on the severity of RH reduction (Ziv *et al.*, 1983; Wardle *et al.*, 1983). Maene and Debergh (1987) and Capellades *et al.* (1990) obtained plantlets with anatomical features comparable to in vivo plants when they reduced RH by bottom cooling to stimulate transpiration.

Short *et al.* (1987) reduced RH in the culture vessel by using different permeable caps. A RH of 80% resulted in normal growth, normal stomatal function, epicuticular wax deposition and reduced water loss with subsequent successful acclimatization of chrysanthemum (*Chrysanthemum xmorifolium* Ram cv. Pennine Reel) and cauliflower (*B. oleracea*). Lower RH of 50% and 30%, however, reduced growth of IVPs (Short *et al.*, 1987). A lid modified by Dillen and Buysens (1989) to increase evaporation promoted dry weights and reduced vitrification of *Gypsophyla paniculata* L.. Fari *et al.* (1987), used thin PVC foil coverage to overcome acclimatization difficulties resulting in vigorous plantlets with increased dry weight, wax profusion and improved hardening.

Tanaka *et al.*, 1988 obtained accelerated shoot development and enhanced growth of *Spathiphyllum sp.* 'Merry' plantlets using gas permeable containers 'Culture Pack' made of fluorocarbon polymer films. Smith *et al.* (1990) reduced the humidity of *C. xmorifolium* cultures to 40% using containers with holes covered by selectively permeable membranes. They obtained plantlets with reduced wilting, thicker leaves, improved stomatal closure and increased cuticle thickness, as well as significantly higher concentration of chlorophyll.

A semipermeable cover (SPC) developed by Kozai *et al.*, (1988), to improve gas exchange, particularly CO_2 for photosynthesis, was reported (together with CO_2 enrichment, supplemental lighting, and sucrose elimination) to improve acclimatization of IVPs. The influence of the SPC on the water status of in vitro cultures and acclimatization of IVPs were not closely examined however. In this study, we investigated the influence of the SPC on some characteristics of IVPs

related to water relations and acclimatization. These include water loss of the tissue culture jars, moisture loss of the plantlets and their detached leaves, epicuticular wax content, Ψ_w of the leaves and media, and plantlet growth and survival in vitro and ex vitro.

Materials and Methods

Plant culture. Tobacco (*Nicotiana tabaccum* L. 'Wisconsin 38') plantlets were grown from single nodal sections cultured on agar-solidified (0.6%) Murashige and Skoog medium (MS; Murashige and Skoog, 1962) with 3% sucrose and no hormones in 200 ml baby food jars. Plantlets were grown in culture for 30 days after which they were transferred to the greenhouse and followed for another 30 days.

Tobacco seedlings were started from seeds germinated and grown for about one month in clay pots on a soil mix of 2 peat : 1 vermiculite : 2 perlite (by volume) in a growth chamber (Perceival, Boone, Iowa) at 25° C and 16/8 light/dark photoperiod of about 170 μmole m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The seedlings were transferred to the greenhouse and watered with Peters 20N-10P-20K fertilizer. Light intensity in the greenhouse ranged between 300 to 800 μmole m^{-2} s^{-1} depending upon clouds. Temperatures and RH in the greenhouse ranged between 20° C to 27° C and 20% to 90% respectively. RH during most of the growth period ranged between 50% and 70%.

Treatments. Two kinds of tissue culture vessel closures were used: B-caps (Magenta Corp., Chicago) and the polypropylene semipermeable caps (SP-caps; Suncap closure; Sigma Chemical Company, Missouri) used by Kozai et al. (1988).

The SP-caps were autoclaved separately with Kimwipe tissue between the sheets and then used to close the sterilized culture jars aseptically upon culture of explants. Greenhouse seedlings were included as a treatment in some measurements for comparison.

Growth Conditions. The culture conditions in the growth room were: 25° ± 3° C on the growth shelves with PPFD of 80 to 90 μmole m^{-2} s^{-1} at the plantlet level in the jar irrespective of closure and a room RH of about 30% ± 5%.

I. Experiment 1. Relative water loss of the medium

In vitro cultures incubated at the above conditions were monitored for water loss of the culture medium by weighing the jars every 48 hours for 30 days. Treatments included the two types of closures with plantlets in cultures or jars with medium only. The relative water loss (RWL) was calculated by the formula:

$$RWL(\%) = \frac{\text{weight loss of the jar}}{\text{initial weight of the jar}} X100$$

The rate of water loss was calculated from the regression of the water loss curve. Plantlet water loss was calculated from the difference between the relative water loss of jars containing plants and those without plants. The rate of water loss for the plantlets was also deduced from the regression formula. Fifteen replicates were used for each of the two treatments with or without plantlets (2X2 factorial) in a randomized complete block design. Analysis of variance and mean separation were done for the four treatments (all combinations) at each time point using General Linear Models and Student Neuman-Keul's mean separation (SAS, 1985).

II. Experiment 2. Relative humidity within vessels

Relative humidity (RH) in the culture vessels was measured for the two closure treatments when plantlets were 3-4 cm in length. The measurements were done using a laboratory made high precision thermocouple psychrometer (copper-constantan; diameter 0.003mm; copper PN:TTCP-003-50ft, constantan PN:TFCC-003-50Ft; Omega Engineering, Inc., Stamford, CT). RH was measured in two experiments: first with non-aspirated thermocouple psychrometers and then with thermocouples aspirated with small fans run by tiny motors (motor mode FA130, CER-MAG motors, Japan). The psychrometer constant was calculated for each case using standard saturated salt solutions of sodium chloride (75.1% RH at 25°C) and potassium sulfate (97.5% at 25°C). Four replicates of each of the treatments were measured in each of the two experiments. The replications were measured for one to two days each. Analysis of variance and mean separation were done using General Linear Models and Student Neuman-Keul's mean separation (SAS, 1985).

III. Experiment 3. Water potential

a. Water potential of the media. Water potential of the medium was measured at the end of the 30-day-growth period to verify agar medium dryness. Water potential was measured using a thermocouple psychrometer in a C-52 sample chamber (Wescor Inc., Logan, Utah) connected to a microprocessor (Model HP-115, Wescor Inc, Logan, Utah). Seven replicates for each treatment were used in a completely randomized design. Analysis of variance and mean separation were

done using General Linear Models and Student Neuman-Keul's mean separation method (SAS, 1985).

b. Water potential of plantlets. The third leaf from the top (most recent fully expanded) was detached immediately after removal from culture and inserted in sample chambers containing a thermocouple psychrometer (chamber 81-500 and chamber psychrometer 83 series, J.R.D. Merrill Specialty Equipment, Logan, Utah) connected to an HP-115 microprocessor (Wescor Inc., Logan, Utah). Six calibrated chambers were loaded with leaves from the two treatments (3 leaves for each treatment). The chambers were kept at a constant temperature of 25° C using a water bath (CH/P temperature control system; bath and circulator model 2067; Forma Scientific / Mallinckrodt, Inc., Marietta, Ohio). The chambers were then dipped in liquid nitrogen and back into the water bath for osmotic potential (Ψ_π) measurement. Pressure potential (Ψ_p) was calculated from the difference between Ψ_ω and Ψ_π. The six chambers were loaded three times on three consecutive days thus forming the nine measurements for each treatment. Analysis of variance and mean separation were done using General Linear Models and Student-Neuman-Keul's mean separation method (SAS, 1985).

IV. <u>Experiment 4</u>. Water loss of detached leaves

Plantlets' leaves were detached immediately after removal from culture and weighed. The leaves were then exposed, abaxial side up, to ambient conditions in the laboratory of 30% RH, 22° C $\pm$ 1° C and 10 μmole m^{-2} s^{-1} PPFD. Leaves were then weighed after 15, 30, 60, 120, and 240 minutes of removal from culture. Dry weights of leaves were taken after oven-drying for 48 hours at 70° C.

Moisture loss was calculated on a dry weight basis according to the following formula:

$$\text{Moisture loss/unit dry weight} = \frac{(Fwt\text{-}Dwt)\text{-}(Fwt_t\text{-}Dwt)}{Dwt}$$

where Fwt = fresh weight, Dwt = dry weight, Fwt_t = fresh weight after time. Moisture loss on a dry weight basis has been found to be a more reliable method of monitoring moisture loss of plants (McCaig and Romagosa, 1989).

Ten replicates were used per treatment in a completely randomized design. Moisture loss data was analyzed and means were separated for each time point using General Linear Models and Student-Neuman-Keul's mean separation method (SAS, 1985). The rate of moisture loss was deduced from the change in water content during a 4-hour-drying period.

V. Experiment 5. Leaf epicuticular wax content

Leaf wax was extracted from detached leaves by dipping them three successive times (30 seconds each) into beakers with 150 ml of chloroform. The extract was then pooled, the beakers rinsed with warm chloroform and poured into a round bottom flask by filtering through anhydrous sodium sulfate placed on top of glasswool. The chloroform was then evaporated using a rotovaporizer (R-Bushi, Rinco Instrument Co.) with a water bath not exceeding 38° C. The last 4 to 5 ml of the extract were left to be transferred into pre-weighed aluminum weighing dishes. The aluminum dishes were left to evaporate in a fume hood. The wax content was then determined gravimetrically and expressed on leaf area basis. Leaf area was measured using LI-COR area meter (model LI-3100, Lambda Inst. Corp., Lincoln, Nebraska).

Ten replicates were used for each treatment. Analysis of variance and mean separation were done using General Linear Models and Student Neuman-Keul's mean separation method (SAS, 1985).

VI. <u>Experiment 6.</u> Growth analysis

Plantlet growth in culture and in the greenhouse was followed for the two treatments by measuring fresh weight, dry weights, number of leaves, leaf area, stem length, root fresh weight and dry weight at 10 day intervals starting 20 days after culture and continuing until 30 days after transfer to the greenhouse. Ten replicates were used for each time point. Analysis of variance of the growth parameters was done for each time point using General Linear Models and Student-Neuman-Keul's test for mean separation.

Relative growth rates (RGR), net assimilation rates (NAR) and leaf area ratio (LAR) were calculated as indices of the efficiency of the leaves in photosynthesis. The formula for their calculations are indicated in the corresponding table. Analysis of variance was conducted using General Linear Models and Student-Neuman-Keul's test for mean separation (SAS, 1985).

Survival and wilting injury of plantlets transferred to the greenhouse were monitored by counting the number of injured leaves and scoring the injury from 1 (least injured) to 5 (most injured). Ten replicates were used for each time point. Analysis of variance of calculated injury was done for each time point using General Linear Models and Student-Neuman-Keul's test for mean separation (SAS 1985).

Results and Discussion

I. Experiment 1. Relative evaporative water loss of the medium

Evaporation of moisture from the culture vessels was determined by calculating relative water loss (RWL) to study the closure effect on water status of the cultures. Vessels covered with SPC had a RWL of about 2.5 times more than B-cap jars by the end of the 30-day culture period (Fig. 5.1).

Plantlet transpiration in the vessel (deduced from the difference in water loss between jars containing plants and those without plants) was similar in the first 14 days of culture after which plantlets from SPC treatments lost more water than plantlets from B-cap closure treatment. However, the difference between closure types was not statistically significant (Fig. 5.2). The non-significance could be due to the indirect method of calculation of plantlet water loss.

The rate of evaporative water loss was 13.2 mg H_2O h^{-1} for jars covered with SPC and was significantly higher ($P < 0.01$; Table 5.1) than that for jars covered with B-caps (5.3 % H_2O hr^{-1}). For both closure treatments plantlet presence in the jar increased the rate of water loss significantly over jars containing only media only and in both closure treatments (Table 5.1, Fig. 5.1).

The demonstrated higher evaporative water loss from the culture vessel associated with the permeability of the SPC could have had one or all the following effects on the plant culture: **1)** activation of transpiration through the continuous water loss stream to the atmosphere, **2)** conditioning of the plantlets to a lower RH or **3)** desiccation of the medium, resulting in low Ψ_w which may further induce acclimatization of plantlets.

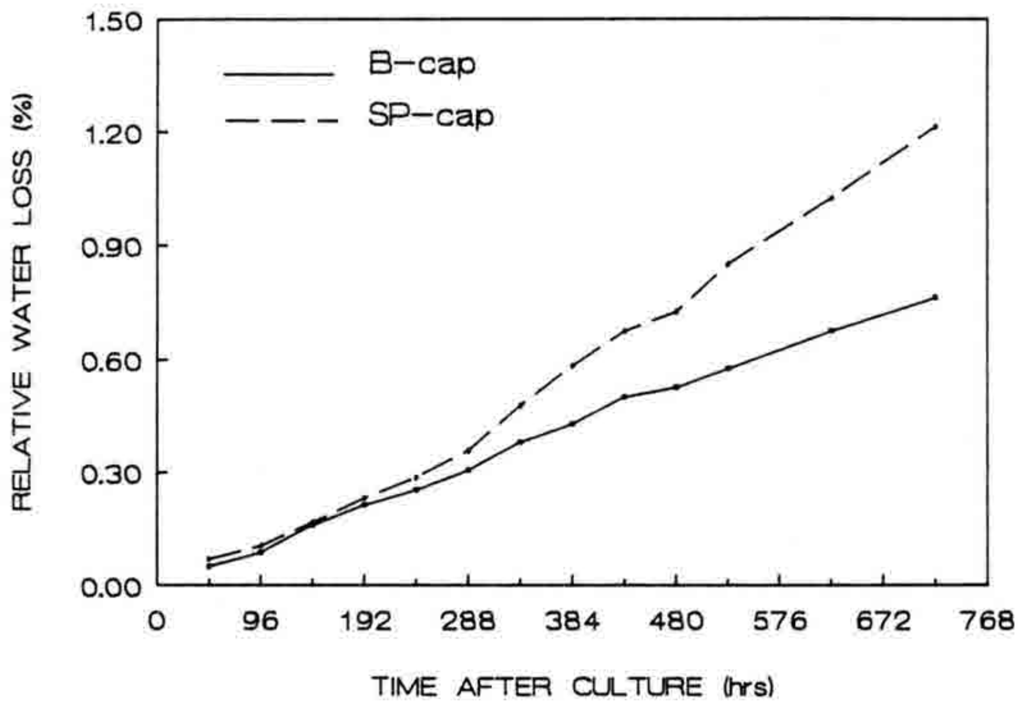

Figure 5.2. Relative water loss of plantlets (%; RWL) grown in vessels covered with B-cap and SP-cap closures.

Table 5.1. Rate of water loss of jars covered with B-cap and SP-cap with and without a plantlet.

Treatment rate of water loss (mg hr^{-1})	B-cap - plantlet 4.7	SP-cap + plantlet 14.1
SP-cap -plantlet 12.3	effect of cap ***	effect of plantlet ***
B-cap + plantlet 5.8	effect of plantlet ***	effect of cap ***

*** highly significant F value ($P < 0.01$)

Activation of transpiration enhances mineral uptake necessary for growth and osmotic adjustment (Pospisilova *et al.*, 1987, Kozai *et al.*, 1988) which may lead to normal development of vascular system and function necessary for normal water uptake upon transplanting to greenhouse conditions (Maene and Debergh, 1987; Marin and Gella, 1987; Kozai, 1988). Normal water transport may also stimulate function of stomates and epicuticular wax deposition to control water loss.

Sutter (1988) and Shackel *et al.* (1990) have demonstrated water uptake of IVPs during acclimatization but plantlets still had slow stomatal functioning. Marin and Gella (1988) demonstrated active water uptake but their plants died after 5 weeks, possibly due to failure of establishment of a successful autotrophic photosynthesis. Water uptake, therefore, is only one of many factors involved in acclimatization of IVPs as reported by Sutter (1988). Our results above show that plantlets in culture with SPCs transpired slightly more (1.2% of their initial weight) than plantlets from B-cap treatments (0.7%) which indicates slightly enhanced

transpiration. Whether this transpiration contributed to acclimatization cannot be answered. However, a small increase in transpiration rate was not viewed as sufficient to induce water stress acclimatization (Kozai, 1988).

II. Experiment 2. Relative humidity within vessels

The RH of vessels covered with B-cap and SPC was always greater than 90%. However, SPC covered vessels had a significantly lower RH averaged over a whole day. The RH of B-cap vessels ranged from 98% to 100% while RH of SPC vessels ranged from 92% to 97%. The daily average RH of the B-cap vessels was 98% and 99% according to two different experiments while SPC daily average RH was between 96% and 97% (Safadi *et al.*, 1992). It is not known, however, whether the reduction in RH due to SPC affected water status of the plantlets and their acclimatization. The increased water loss of the plantlets in SPC covered jars after 14 days of culture may be the result of the reduced RH, thus indicating active transpiration. However, the RH that we observed in the SPC covered vessels was relatively high. At times, we observed condensation of water in the jars of both kinds of closures indicating RH of 100%. The condensed water in the SPC covered vessels, however, dried out faster than that in B-cap vessels, thus demonstrating higher evaporation rate for SPC vessels. In addition, our measurements of RH were done in a period (after two weeks of culture) when culture media had high water availability thus SPC reduced RH only slightly. This appears consistent with a previous report in which low RH levels in culture vessels were not attainable over liquid media by using a desiccant, while RH was reduced to 85% over agar media

(Ziv *et al.*, 1983). Maene and Debergh (1987) could not reduce RH significantly below 100% by using different permeable caps, yet their media desiccated.

The need to reduce RH of the vessel to induce acclimatization in vitro has been realized with early acclimatization studies (Sutter and Langhans, 1982; Ziv *et al.*, 1983). The consensus was that reducing RH in the culture environment is the key factor for reducing vitrification (Short *et al.*, 1987). Successes were obtained in inducing stomatal closure, epicuticular wax deposition, reducing water loss, obtaining glaucous leaves and improving survival by exposing the plantlets to reduced RH in vitro (Brainard and Fuchigami, 1981; Wardle *et al.*, 1983; Ziv *et al.*, 1983; Short *et al.*, 1987; Maene and Debergh, 1987; Capellades *et al.*, 1990; Smith *et al.*, 1990). Low RH, however, in some of the above research (Wardle *et al.*, 1983; Ziv *et al.*, 1983; Short *et al.*, 1987; Dillen and Buysens, 1989; Maene and Debergh, 1987) drastically affected shoot growth and multiplication.

The methods used to control RH such as desiccants (Ziv *et al.*, 1983), lanolin overlays (Wardle *et al.*, 1983; Crane and Hughes, 1990) and lose fitting or gas permeable caps (Short *et al.*, 1987; Dillen and Buysens, 1988; Maene and Debergh, 1987, Hakkaart and Versluijs, 1983) caused drastic changes in RH with adverse effects on growth. Reducing RH by different permeable caps resulted in dehydration of the agar medium although RH was not reduced considerably below 100% (Maene and Debergh, 1987). This caused salt accumulation in the medium which affected root growth and limited the use of the system to a maximum of 10 days. Reducing RH through increasing agar concentrations inhibited root growth and did not improve survival (Rahman and Blake, 1988). Methods such as bottom

cooling provided good control over RH without affecting growth improved acclimatization (Maene and Debergh, 1987; Capellades *et al.*, 1990). Dillen and Buysens (1989) reduced RH by a lid modified to be semipermeable, but used it for a short period to avoid medium drying and its associated effects on growth. Our method of reducing RH by SPCs also caused medium desiccation if used for a prolonged time (Fig. 5.3). However, it did not reduce RH so drastically as to affect growth.

The differential rate of evaporation between B-cap and SPC vessels and medium dehydration in time may result in further reduction in RH of the vessels (Fig 5.3). In theory (Raoult's Law), vapor pressure over perfect solutions is proportional to the mole fraction of the solvent (Salisbury and Ross, 1985).

Although agar medium is not a perfect solution, its Ψ_ω is reduced, which affect RH of the closed container. Ziv (1986) reported that the effect of increased agar concentrations on acclimatization is primarily through its effect on reducing RH in the culture vessels. Although we did not measure RH at the end of the 30-day-culture period, the Ψ_ωs of the media and the leaves were measured which gives us an idea of the water status of the culture.

III. Experiment 3. Medium and leaf water potential of the media

The Ψ_ω of the medium in SPC covered jars at the end of a 30-day-culture period was significantly lower (-14.4 bars; $p < 0.01$) than that of the B-cap covered jars (-4.5 bars; Fig. 5.4). This is a further evidence of medium drying due to evaporation as related to the SPC closure. Dillen and Buysens (1990) actually calculated media drying from the evaporative weight loss of the vessels.

Figure 5.3. Tobacco tissue culture vessels covered with the plastic B-cap (left) and semipermeable cap (SP-Cap; right) showing desiccation of the media after six weeks of culture.

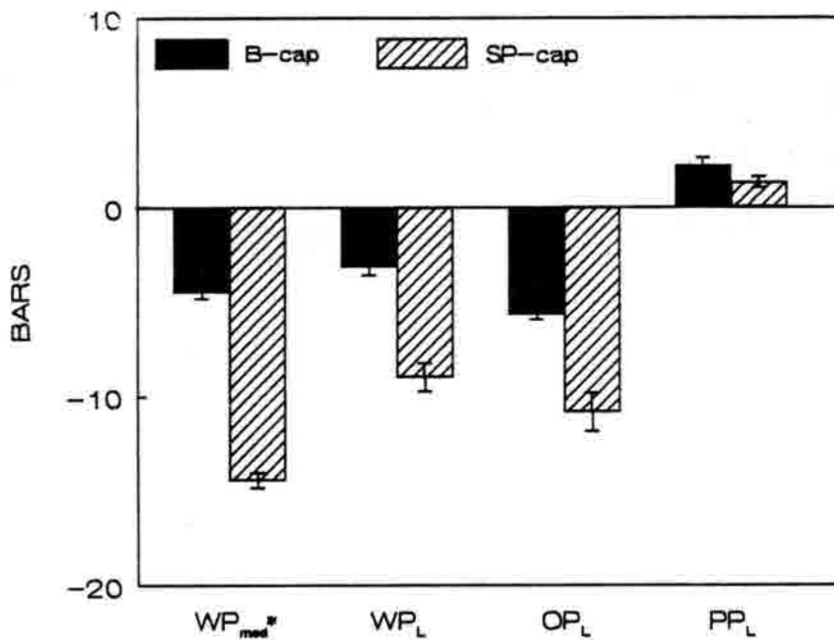

Figure 5.4. Water potential of the growing medium (WP_{med}) and Water (WP_L), osmotic (OP_L), and pressure (PP_L) potential (bars) of leaves of in vitro plantlets after one month growth period in vessels covered with B-cap and SP-cap closures. Vertical bars indicate $\pm$ standard error. *medium water potential was determined in a separate experiment from that for leaf water potential.

The main effect of low Ψ_w on acclimatization is thought to be through a reduction in the water status of the cultures, both plantlets and culture environment (Debergh *et al.*, 1983; Ziv, 1986; Rahman and Blake, 1988).and Blake, 1988). A Ψ_w of -14 bars in a closed container would equilibrate a RH of 98.98% at 25° C, while a Ψ_w of -5 bars would result in RH of 99.64%. Thus Ψ_w of the medium may have little effect on RH of the vessels. It takes a gram-molecular weight of sucrose (342.3 grams) in in a litre of water to reduce RH in a closed container from 100% to to 98.23% (Salisbury and Ross, 1985). However, the tissue culture vessels covered with SPC, are not completely closed, hence RH in these vessels are unpredictable.

Reducing Ψ_w of the medium was reported as a successful method of acclimatization in many plant species (Ziv *et al.*, 1983; Ziv, 1986; Wardle *et al.*, 1983; Short *et al.*, 1987; Leshem, 1983; Debergh *et al.*, 1983). It was observed that in long term cultures the newly formed leaves became more glaucous as the media dehydrated (Ziv *et al.*, 1983). Medium dehydration was also obtained in modified cap treatments which reduced humidity of culture vessels to eliminate vitrification in *G. paniculata* (Dillen and Buysens, 1989). Sutter and Langhans (1982) also suggested that the effect of a reduced RH of 35% on plantlets was through medium desiccation. Water stress of the growing medium is known to improve the adaptation of plants to xerophytic conditions (Dale, 1982; Daly, 1964; Manning *et al.*, 1977; Bengtson *et al.*, 1978).

Reduction of Ψ_w of the media by increasing agar concentrations induced normal glaucous carnation (Ziv *et al.*, 1983), and artichokes (Debergh *et al.*, 1981),

reduced water loss of detached leaves, increased wax deposition and induced normal stomatal functioning in cauliflower and carnations (Wardle *et al.*, 1983; Ziv *et al.*, 1983; 1987). A reduction in Ψ_w of the medium by adding polyethylene glycol to the rooting medium reduced water loss of detached leaves, and increased wax formation, in date palms (Zaid *et al.*, 1989), grape (Dami, 1991) and tobacco (Chapter III). It has also induced stomatal closure in tobacco (Safadi *et al.*, 1990), and resulted in more normal leaf anatomy and improved survival in grapevines (Dami, 1991). However, in most of these studies low Ψ_w of the medium drastically reduced growth which offset the beneficial effects (Ziv *et al.* 1983; Wardle *et al.*, 1983; Dami, 1991; this issue, Chapter III). High agar concentrations used by Rahman and Blake (1988) to control RH affected roots and did not improve the rate of survival after transfer to ex vitro conditions.

Plantlets in high agar concentrations are hardened by exposing their root system to lower Ψ_w in the medium as well as their leaves to lower humidity or Ψ_w of the atmosphere. This treatment may present stressful situation of reduced Ψ_w at the two extremities of the plantlets, thus presenting a dilemma for the plantlets to cope with.

In normal in vivo situations, a gradient of Ψ_w between the air, plant and the medium exists to maintain a driving force for transpiration (Jarvis and Morison, 1981). For example, Ψ_w of a well-irrigated soil is as high as -2 to -3 bars and that of the atmosphere is much lower (RH of 90% is -150 bars, -42 bars for RH of 97%, and -14 bars for RH of 99%) providing a large gradient in Ψ_w for water transport to take place (Pospisilova et al, 1987; Kozai, 1988). Under normal growing

conditions, a gradient in Ψ_w between the soil and the leaf is 3 to 30 bars while the gradient between cells within the leaf and the atmosphere may be about 900 bars (50% RH).

In in vitro plantlets, however, Pospisilova *et al.*, (1987) and Kozai *et al.* (1986) reported that the culture medium Ψ_w is relatively low due to the presence of salts and sucrose in the medium (-5 bars for M&S medium). Plantlet Ψ_w is reduced to levels equal to or slightly below that of the medium. In addition, the high relative humidity in the culture vessel (-14 bars for RH of 99%) provides a very small gradient in Ψ_w (about 10 bars only) for transpiration. Plantlets under such conditions had little transpiration rates and maintained Ψ_w, Ψ_π and Ψ_p much higher than that for seedlings (Kozai 1988; Pospisilova *et al.*, 1988). This reduced transpiration and water uptake were reported to be the cause for the physiological disorders of plantlets (Kozai, 1988; Maene and Debergh, 1987). It has been shown that plants growing in low Ψ_w treatments underwent guttation when they were exposed to high humidity (Kaufmann and Eckard, 1971).

On the other hand, when low Ψ_w is applied to the medium and the RH is reduced, the gradient is further disturbed and water transport is unpredictable. Thus, careful studies of water relations of plantlets in vitro are important in understanding the behavior of IVP under water stress acclimatizing conditions. This would be helpful in designing water stress hardening procedures which are closer to normal conditions and that would not affect growth of IVPs.

There has been limited research on water status of IVPs and their relation to medium and atmosphere water status. Ziv *et al.* (1983) reported a two-fold

increase in Ψ_ω of glaucous plantlets grown under high agar concentrations, but did not study their water transport. Pospisilova *et al.* (1987) noted that due to the low transpiration rate of tobacco plantlets under very high air humidity inside the vessels, the leaf Ψ_ω, Ψ_π and Ψ_p were higher than those for seedlings in spite of a lower Ψ_ω of the medium than that for soil substrate.

Our results relative to Ψ_ω of the tobacco plantlets show a similar trend to those of Pospisilova *et al.* (1987; 1988). Plantlets grown in B-cap covered jars (RH 98 to 100%) had a significantly higher leaf Ψ_ω (-3.1 bars), Ψ_π (-5.7 bars) and Ψ_p (2.2 bars) than those for plantlets grown in SPC covered jars (-8.98, -10.82, 1.3 bars respectively (Fig. 5.4). The lower Ψ_ω in SPC plantlets is due to less available water in the medium as well as the atmosphere. Water potential Ψ_π, and Ψ_p of SPC plantlets fell within the range of that for tobacco seedlings (-8.2, 9.8, and 1.32 bars respectively) measured by Pospisilova *et al.* (1987).

The relationship between leaf Ψ_ω and its components, Ψ_π and Ψ_p, and the medium may indicate the water status of IVP (Fig. 5.5). In both treatments, B-cap and SPC, Ψ_ωs of the leaves of plantlets were higher than the medium Ψ_ω. This seems unreasonable since it may imply lack of water uptake or even reversal of water transport from the plant to the medium. However, this abnormality may be an indication of an abnormal physical situation of in vitro cultures. Another possible explanation may lie in the experimental procedure used to measure the medium Ψ_ω. The procedure used a different instrument from that used for the leaves. The leaves also did not come from the same set of cultures as the cultures

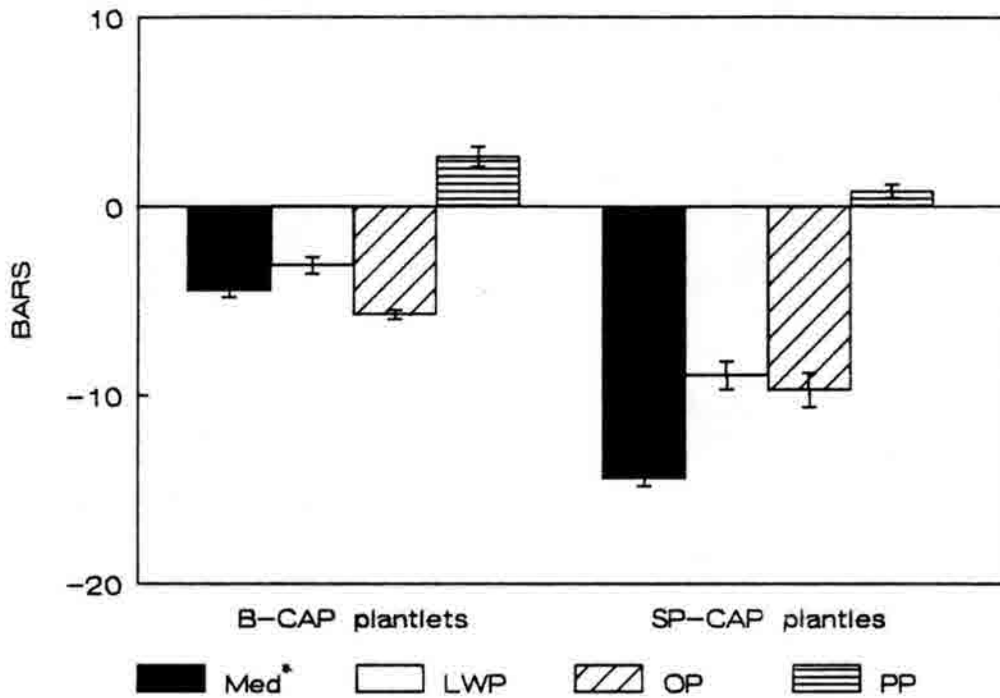

Figure 5.5. Water potential of medium (MED), leaf water potential (LWP), Leaf osmotic (OP), and leaf pressure potential (PP; bars) of in vitro plantlets after a one month growth period in vessels covered with B-cap and SP-cap closures, showing the relationship between Ψ_w and its components. Vertical bars indicate $\pm$ standard error. *medium water potential determined in separate experiment from that for leaf water potential.

measured for the medium Ψ_{ω}. Thus the growth conditions might have been different.

Pospisilova *et al.* (1987) measured a very small difference in Ψ_{ω} between in vitro tobacco and the medium and described it to be too small to cause efficient water transport. Small driving force for transpiration resulting from low Ψ_{ω} gradient (10 bars only) between air (-14 bars at 99% RH) and salt medium (-5 bars for M&S medium) was also reported (Kozai, 1988). Our results for B-capped culture vessels which exhibit high Ψ_{ω} and lack a gradient in Ψ_{ω} between the medium and leaves could be explained similarly because of the high humidity in the B-cap covered culture vessel and errors normally involved with Ψ_{ω} measurements.

Turgor pressure was high in plantlets grown in B-cap vessels due to low transpiration created by the high relative humidity in the culture vessels. This type of relationship is typical of in vitro cultures (Pospisilova *et al.*, 1988; Kozai, 1988; Ziv *et al.*, 1983). Osmotic adjustment of in vitro tobacco leaves was reported by Pospisilova *et al.* (1988) where a correlation was found between Ψ_{ω} of in vitro tobacco leaves and their Ψ_{π} demonstrating that the low Ψ_{ω} and the maintenance of turgor were due to osmotic adjustment in plantlets (Pospisilova *et al.*, (1988).

Plantlets grown in SPC covered jars have abnormal Ψ_{ω} relationships with the medium similar to those found in B-cap plantlets (Fig. 5.4.). In spite of the indication of abnormal physical condition in the culture environment, we will ignore medium Ψ_{ω} because of procedural suspicions mentioned earlier. Leaf Ψ_{π} of plantlets grown in SPC covered vessels decreased slightly to maintain some turgor pressure. The turgor was less than but insignificantly different from that of B-cap

plantlets. The low Ψ_w in SPC plantlets seemed to be due to low turgor as well as osmotic adjustment. Pospisilova *et al.*, (1988) found similar findings where the seedlings' low Ψ_w was due to lowered turgor pressure rather than to lowered Ψ_π as found in their in vitro counterparts.

The relationship of the SPC plantlet's water relations with the atmosphere and medium could not be explained due to lack of RH measurements at the end of the culture period and a corresponding medium Ψ_w. However, SPC plantlets managed to maintain normal turgor (comparable to seedlings) under the water stress conditions in the medium and the atmosphere (assumed to be 96 to 97% RH or in the vicinity of -42 bars as in Kozai, 1988). Under such assumptions (medium Ψ_w -14.4 bars and atosphere Ψ_w of -42 bars) a gradient for water transport of 27.6 bars existed. Such gradients were not enough for maintaining a normal soil-atmosphere continuum (Kozai, 1988).

It is known that plants thriving in very dry areas must have efficient systems that balance water uptake and loss (McCaig and Ragamosa, 1989). How the SPC plantlets managed to keep turgor is unknown but their water stress resistance seemed to involve tolerance of the physiological processes to low water and Ψ_p. In another part of our study we demonstrated possible hardening of the photosynthetic system to water stress (Safadi *et al.*, 1991). Osmotic adjustment may have taken place as well as possible control over water loss through stomatal closure or surface wax. Water potential of plantlets of SPC covered jars is in the normal range of in vivo field crops growing on well irrigated soils which is reported to range between -9 to -13 bars at midday (Hsiao and Bradford, 1983). The reduced Ψ_w of the

plantlets is advantageous upon transfer to soil since it stimulates water uptake due to the greater Ψ_w gradient between soil and leaves (Pospisilova *et al.*, 1988). Therefore, SPC plantlets are induced to be water stress resistant since they maintained turgor pressure similar to that of seedlings. The mechanism of resistance is unknown but a review of the features of acclimatization in the next sections may offer some ideas on the resistance of SPC plantlets.

IV. <u>Experiment 4.</u> Moisture loss of detached leaves

Brainerd and Fuchigami (1981) suggested that rate of moisture loss (RML) of detached leaves can be used as an index of acclimatization of IVPs. Detached leaves of in vitro tobacco plantlets grown in SPC covered vessels lost significantly less water ($P < 0.01$) than those grown in B-cap covered jars when dried at 30% RH (Table 5.2). The leaves from SPC treatment had a significantly lower RML (0.803 g H_2O g^{-1} dry weight hr^{-1}; $P<0.01$) than that of leaves of plantlets grown in B-cap covered jars (1.706 g H_2O g^{-1} dry weight hr^{-1}, Table 5.2). Leaves from B-cap plantlets lost 8.7% of their water content in the first 15 minutes as compared to 6% lost from leaves from SPC plantlets (Table 5.2). Leaves from B-cap plantlets lost 60% of their water content after a 4-hour desiccation period, which was significantly higher ($P < 0.01$) than water lost by leaves from SPC plantlets (47%). Plant cells are reported to be damaged when they lose about 50% of their water content (Kobayashi *et al.*, 1981).

Water loss of detached leaves calculated on dry weight basis (Fig. 5.6) shows that the reduction in water loss by SPC treatment is even more substantial. SPC therefore has limited some wilting injury by reduction of water loss during the first

Table 5.2. Moisture loss (% ML) and rate of moisture loss (RML) of detached leaves and leaf surface wax content of plantlets grown in B-cap and SPC covered vessels and greenhouse plants (GH).

	% ML at time after detachment (min.)						
Treatment	15	30	60	120	240	RML g H_2O g dwt^{-1} h^{-1}	Wax Content mg cm^{-2}
B-cap	8.67 a	16.04 a	25.13 a	40.07 a	59.73 a	1.706 a	0.164 b
SP-cap	5.92 b	9.82 b	16.80 b	28.20 b	46.78 b	0.803 b	0.251 a
% change	32	39	33	30	22	45	35
GH	5.97	9.63	12.32	15.03	19.98	0.695	0.069

Means followed by the same letters are not significantly different at $P < 0.01$.

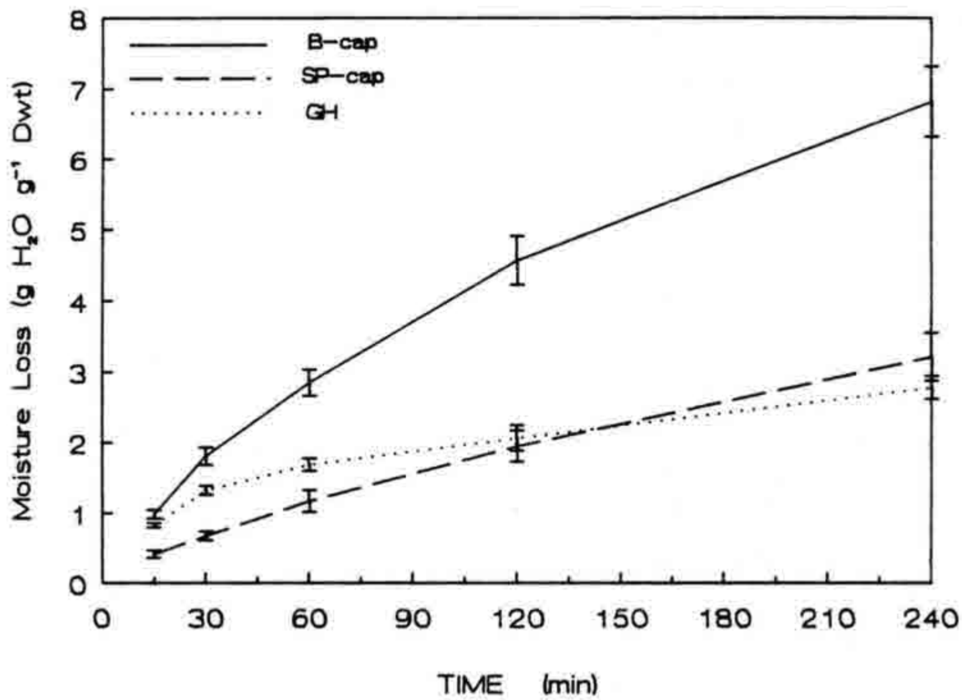

Figure 5.6. Moisture loss per unit dry weight (Dwt) of detached leaves of in vitro tobacco plantlets grown for one month in vessels covered with B-cap and SP-cap closures, and greenhouse grown plantlets, vertical bars indicate $\pm$ SE.

four hours (Fig. 5.7). Water loss as determined by dry weight basis gives a more realistic idea of behavior of plants in determined by dry weight basis gives a more realistic idea of behavior of plants in terms of water loss (McCaig and Romagosa, 1989). Water loss per unit dry weight of plantlets was reduced by 57%, 63%, 59%, 57%, and 52% in leaves from SPC plantlets over those for leaves from B-cap plantlets as they air dried for 15, 30, 60, 120, and 240 minutes respectively. Such a reduction in water loss may be significant in protecting the plantlets from injury effects associated with rapid water loss upon transfer to the greenhouse.

It is known that immediately after detachment leaves lose water rapidly through open stomates (McCaig and Romagosa, 1989). As stomates close, water loss quickly declines until the rate becomes linear within approximately 30 minutes of excision and remains constant for several hours. The initial water loss is due to stomatal transpiration, with subsequent loss associated with cuticular losses (McCaig and Romagosa, 1989). The shape of the water loss curve for our results does not show the typical stomatal and cuticular phases as demonstrated for greenhouse plantlets (Fig. 5.6). This indicates ineffective stomatal control of water vapor efflux in both treatments.

Water loss of leaves from the SPC treatments is within the range of that for the greenhouse seedlings. However, the curve's shape is different and water loss continues to increase for the 4 hours of drying. Water loss is initially (at 0 time) lower for leaves from SPC vessel grown plantlets than B-cap treatment. Thus, the difference in water loss between the two treatments may be due to differences in

Figure 5.7. Detached leaves of tobacco plantlets grown in culture vessels covered with the plastic B-cap (left) and the semipermeable (SP-cap) covers (right) showing differences in wilting after four hours of air drying at 30% RH.

initial stomatal apertures, epicuticular wax contents and a possible overall change in anatomy which increases the resistance of the leaves to water loss.

V. <u>Experiment 5.</u> Epicuticular wax content

Although wax content is significantly higher ($P < 0.01$) in plantlets from SPC covered jars (0.251 mg cm^{-2}) than those in plantlets from B-cap covered jars (0.164 mg cm^{-2}; Table 5.2), wax content is reported to be insufficient for controlling water loss, especially if the stomates are open (Conner and Conner, 1984, Sutter 1985, Brainerd and Fuchigami, 1981). In addition, wax contents of the greenhouse grown leaves were very low (0.069 mg cm^{-2}), yet their water loss was lower than IVPs in B-cap covered vessels. This result agrees with Sutter's (1985) findings where in vivo plantlets did not always contain higher wax content than in vitro plantlets. In addition, epicuticular wax contents did not correlate with rate of survival (Sutter, 1985). Several other authors could not find a straight relationship between rate of water loss and wax content (Bengtson *et al.*, 1978, Denna, 1970) although increased wax contents was associated with glaucousness (Denna, 1970), xerophytic ecosystems (Daly, 1964) and water stress treatments (Bengtson *et al.*, 1978).

It is possible that the reductions in water loss in SPC treated leaves have arisen from differences in the development of stomates like frequency or size of apertures due to the unusual water stress conditions that they grew in. Wardle *et al.*, (1983) found changes in the development of stomates influenced by severe water stress treatments. Brainerd and Fuchigami (1981) found differences in water loss between greenhouse seedlings and in vitro plantlets, although they were at the

same level of stomatal closure. They explained the difference by possible wax increase or stomatal development and frequency.

Reducing Ψ_w of the media reduces transpiration (Janes, 1966). Stomates remained closed as long as the low Ψ_w was maintained (Janes, 1966). Stomates of plants suffering from water stress respond by closing (Losch and Tenhunen, 1981). The closure results from general turgor loss of the leaf which affects guard cells and may stimulate abscissic acid formation. This is a feedback mechanism which includes the effect plant water status has on stomatal closure (Losch and Tenhunen, 1981). Under these conditions, stomata may remain closed even during the light period (Davies *et al.*, 1981). Under intermediate levels of stress, the degree of water deficit influences stomatal response to photon flux density, temperature, carbon dioxide and vapor pressure deficits and establishes a ceiling for stomatal aperture (Davies *et al.*, 1981). Increasing the levels of stress will result in greater limitation of the maximum degree of stomatal opening (Davies *et al.*, 1981).

It appears, therefore, that low water status, due to SPC, of the tobacco cultures reduced stomatal aperture and reduced initial water loss. However, this is different from the direct stomatal response to atmospheric vapor pressure deficits which is mediated by hydropassive mechanism between guard cells and epidermis (Lange *et al.*, 1971). This feedforward mechanism is the one which signifies the normal stomatal functioning in reducing transpiration as evaporative demand increases (Losch and Tenhunen, 1981). Our experiments did not detect this type of response but the feedback mechanism which could be advantageous in reducing transpiration upon transplanting IVPs, but may be not normal.

The reduced water loss, stomatal closure and increased epicuticular wax content encountered in plantlets with SPC treatment are similar to results obtained by several others who induced acclimatization by reducing water status of the cultures (Wardle *et al.*, 1983; Ziv *et al.*, 1983, Dillen and Buysens, 1989; Fari *et al.*, 1987; Crane and Hughes, 1990; Smith *et al.*, 1990; Capellades *et al.*, 1990; Zaid and Hughes, 1989). However, except for an attempt by Ziv *et al.* (1983), none of these studies explained the association between their treatment effect on water relations of the plant and plantlet modifications as related to acclimatization.

Another moisture loss experiment was done in which cultures with SPC were corrected for the Ψ_w of the medium by adding sterile water daily to gravimetrically match that of B-cap cultures. The cultures were grown for one month. Reduction in water loss of detached leaves was still induced by the SPC treatment but to a lesser extent than in the previous experiment (Fig. 5.8). This shows the significance of the evaporation-induced-water stress applied by medium evaporation in inducing hardening. Another factor, however, is involved in inducing a small degree of water conservation but is less effective than medium Ψ_w. In this experiment, the RH of the vessel was not measured but assumed to be close to a 100% since there was more water available in the medium and we could see condensed water droplets. It is possible that the improved gas exchange (CO_2 concentration) provided by the SPC improved dry weights and enhanced growth, thus increasing the degree of control over water loss. We calculated significantly larger dry weight:fresh weight ratios ($P < 0.01$) for plantlets from SPC covered vessels than those from B-capped vessels (results not included). Such a result was reported by Kozai (1988), where

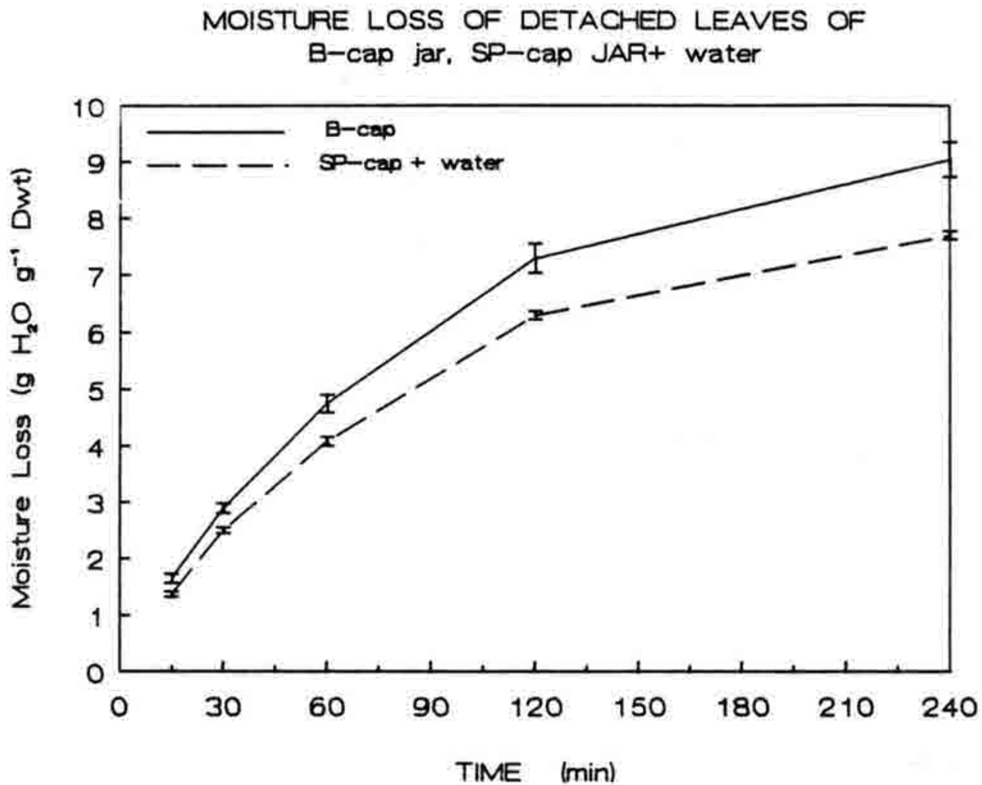

Figure 5.8. Moisture loss per unit dry weight (Dwt) of detached leaves of in vitro tobacco plantlets grown for one month in vessels covered with B-cap and SP-cap closures. Cultures with SP-cap were replenished for the evaporated water content to the same level of that of B-capped vessel. Vertical bars indicate ± Standard Error.

improved biomass of in vitro cultures were noted to enhance acclimatization. Fari *et al.*, (1987) also associated improved dry weights with hardening of tissue culture plantlets.

VI. <u>Experiment 6.</u> Growth Analysis and transplanting injury

Tobacco plantlets from both closure treatments were found to survive the transplant shock in the greenhouse. Although moderate to severe stress was observed with death of some leaves in plants from B-capped vessels (Fig. 5.9), transferred plantlets produced new leaves and continued growth. In addition, after a period of about 20 days of growth, the lower leaves became senescent while new growth flourished. For this reason, a survival study for tobacco plantlets was not possible. Instead, a growth analysis study was done to examine growth in vitro as affected by the closure treatment. Growth was also followed after transfer to the greenhouse to study subsequent effects of the closures on growth and injury of plantlets in the greenhouse. Time courses for fresh weights, dry weights, leaf area, number of leaves and stem length were studied (Table 5.3). Two periods of growth were followed: 1) in vitro-20 and 30 days after culture and 2) ex vitro-40, 50 and 60 days after culture. The injury of plantlets was scored at each time and compared (Table 5.3). In addition, the number of new developing leaves were counted after 50 days and 60 days (Table 5.3).

At 20 days of age, in vitro plantlets from B-cap treatments showed larger shoot fresh weight, stem length and leaf area (Table 5.3). No differences in dry weights were observed. This increased growth in terms of fresh weight was typical of plants grown under high humidity (Cutter, 1971) as expected in B-cap covered

Figure 5.9. In vitro tobacco plantlets grown in vessels covered with plastic B-cap (left) and semipermeable SP-cap (right), **a)** immediately after removal **b)** 15 minutes **c)** 30 minutes **d)** 60 minutes and **e)** after 24 hours of transfer to ex vitro conditions showing rapid wilting in B-cap plantlets and partial recovery after 24 hours.

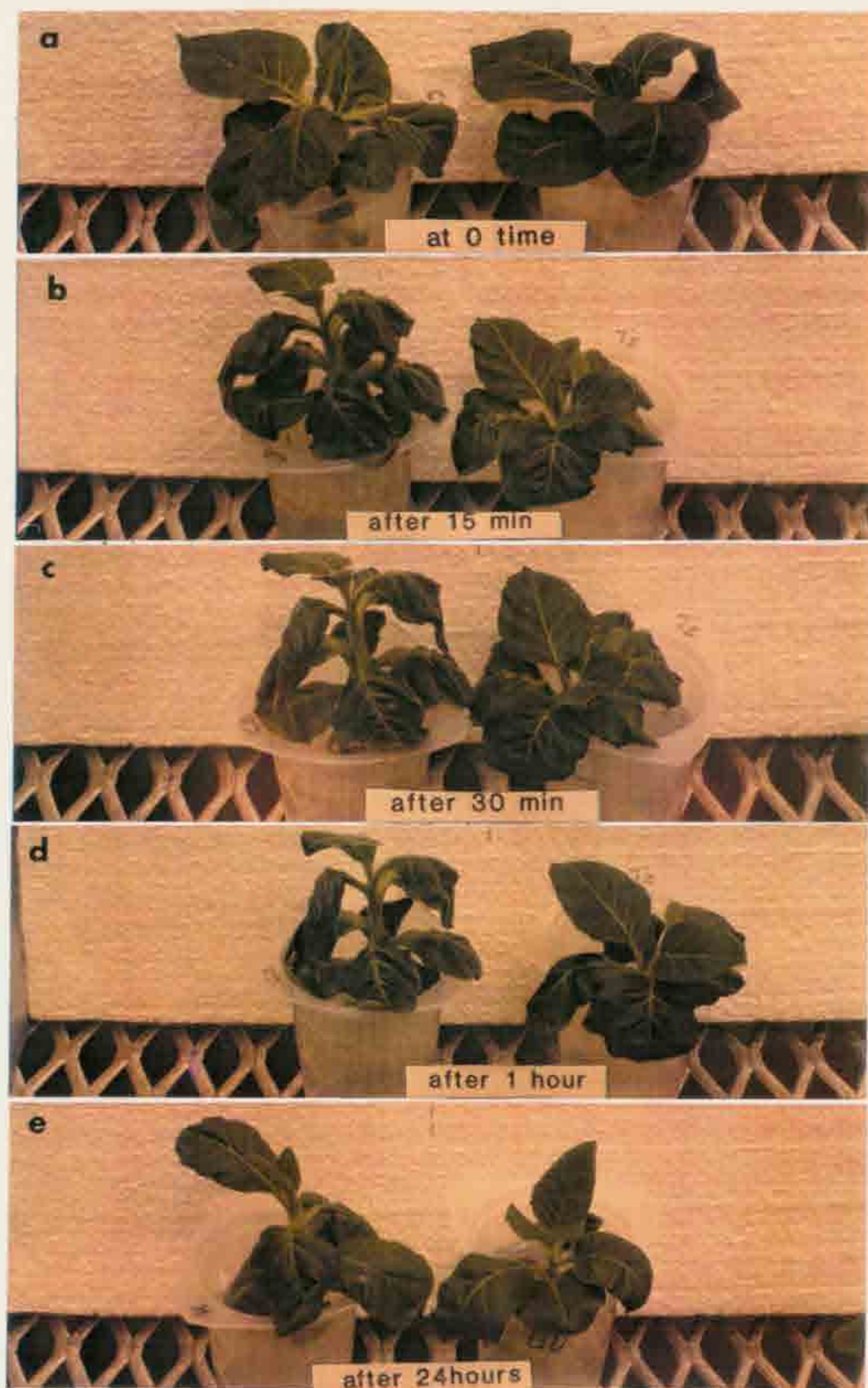
a
at 0 time
b
after 15 min
c
after 30 min
d
after 1 hour
e
after 24hours

Table 5.3. Growth of tobacco plantlets grown in vessels covered with B-caps and SPC treatments on the 20th, 30th, 40th, 50th and 60 days after culture.

Days after culture	Treat-ment	Fresh Weight (g)		Dry Weight (g)			Leaf area (cm^2)	No. of leaves	Stem length (cm)	% new leaves	Leaf[1] Damage
		shoot	total[2]	shoot	root	total					
20 (in vitro)	B-cap	2.35 a		0.140 a	0.037 a	0.177 a	69.96 a	9.60 a	3.13 a		
	SP-cap	1.81 b		0.138 a	0.039 a	0.177 a	59.24 a	8.90 a	2.47 b		
	P-value	0.0196		0.911	0.7285	1.0	0.0667	0.1278	0.0424		
30 (in vitro)	B-cap	3.47 a	4.88 a	0.207 b	0.056 b	0.263 b	109.87 a	10.60 a	3.70 a		
	SP-cap	3.25 a	4.79 a	0.262 a	0.076 a	0.338 a	101.94 a	10.50 a	4.51 a		
	P-value	0.4358	0.8323	0.0116	0.0383	0.0138	0.20	0.7222	0.1011		
40 (10 days in vitro)	B-cap	3.72 b		0.299 b	0.045 b	0.335 b	94.76 b	10.80 a	5.04 a		1.780 a
	SP-cap	4.68 a		0.387 a	0.082 a	0.469 a	120.37 a	11.70 a	5.81 a		1.270 b
	P-value	0.0107		0.0009	0.0001	0.0002	0.0079	0.0965	0.1455		0.0001
50 (20 days ex vitro)	B-cap	10.08 a	13.285 a	0.682 a	0.121 b	0.803 b	296.88 a	15.90 a	160.20 a	44.90 a	3.474 a
	SP-cap	10.16 a	14.327 a	0.808 a	0.168 a	0.976 a	328.02 a	15.70 a	10.45 a	48.40 a	1.457 b
	P-value	0.9325	0.3807	0.0723	0.0078	0.0416	0.1538	0.7372	0.7718	0.1433	0.0001
60 (30 days ex vitro)	B-cap	18.295 a	24.08 a	1.818 a	0.319 a	2.137 a	481.48 a	19.33 a	17.74 a	44.17 a	3.895 a
	SP-cap	17.196 a	22.8 a	1.517 a	0.329 a	1.942 a	457.52 a	16.78 b	18.08 a	50.18 a	2.796 b
	P-value	0.3683	0.3978	0.0566	0.7133	0.1131	0.4689	0.0451	0.7136	0.0514	0.0002

[1] Calculated by multiplying the number of leaves damaged by degree of damage, adding them and then dividing them over their number (weighted average). The degree of damage ranged from 0 to 5 ; 0 = not injured, 5 = most injured.

[2] Root fresh weight was not measured for 20 and 40 days after culture. Thus, total fresh weight is missing.

*Means that are followed by the same letter are not significantly different at the mentioned probability.

vessels. Growth and multiplication have been found to be greater in humid plant cultures of several plant species as compared to those grown under low RH (Wardle *et al.*, 1983; Short *et al.*, 1983; Ziv *et al.*, 1983; Maene and Debergh, 1987; Dillen and Buysens, 1989; Fari *et al.*, 1987). The lack of significant differences in dry weights (Table 5.3) and the significantly lower ($P < 0.01$) leaf dry weight to fresh weight ratio in B-cap plantlets (Table 5.4) indicated that growth increment in fresh weight in B-cap plantlets was due to water retention. By 30 days, plantlets from SPC treatments had significantly larger dry weights of shoots (1.265x), roots (1.35x) and total dry weights (1.285x). The superiority of SPC in accumulating dry weights shows more clearly in Table 5.4 where dry weight to fresh weight ratio, indicative of assimilation (Kozai, 1988) and complex leaf structure (massiveness; Evans, 1972) was significantly larger for SPC plants on day 20 and 30 after culture.

Leaf area ratio (LAR) in Table 5.4 is also an expression of assimilation indicating the leaf area produced per gm dry weight of the plant (Lasko *et al.*, 1986) and leaf structure (Evans, 1972). The lower LAR indicates that plants were growing with less leaf area per unit dry weight. Thus leaves with lower LAR are usually more massive, contain more lignin or store more starch and sugars and may be advantageous in water stress tolerance (Evans, 1972). Lower leaf areas also undergo lower water loss (Lakso *et al.*, 1986). Plantlets from SPC treatment had significantly lower LAR as measured on the 20th and 30th days of in vitro culture (0.85x and 0.72x respectively). This is significant for SPC treatment in that it was somewhat indicative of improved acclimatization upon transfer to the greenhouse.

Table 5.4. Growth ratios of tobacco plantlets grown in vessels covered with B-cap and SPC closures on the 20th, 30th, 40th, 50th and 60th day after culture.

Days after culture	Treatment	Root:Shoot Ratio (dwt basis)	Dry Weight/Fresh Weight			Leaf area ratio cm^2 per g dry wt
			Leaf	Shoot	Total	
	B-cap	0.263 a	0.0609 b	0.0598 b		403.81 a
20	SP-cap	0.276 a	0.0768 a	0.0755 a		346.44 b
	P-value	0.3559	0.0005	0.0005		0.0255
	B-cap	0.268 a	0.058 b	0.0593 b	0.0536 b	429.53 a
30	SP-cap	0.286 a	0.082 a	0.0827 a	0.0721 a	305.97 b
	P-value	0.4202	0.0001	0.0001	0.0001	0.0001
	B-cap	0.1548 b	0.0801 a	0.079 a		284.75 a
40	SP-cap	0.2136 a	0.080 a	0.0833 a		257.70 a
	P-value	0.0006	0.9771	0.3047		0.1074
	B-cap	0.1765 b	0.0674 b	0.0675 a	0.0601 a	374.64 a
50	SP-cap	0.2089 a	0.0743 a	0.0737 a	0.0644 a	341.16 a
	P-value	0.0338	0.0486	0.0824	0.1169	0.1012
	B-cap	0.1776 a	0.0995 a	0.0997 a	0.089 a	225.58 a
60	SP-cap	0.1944 a	0.094 a	0.0965 a	0.088 a	234.01 a
	P-value	0.2409	0.344	0.5243	0.7767	0.5006

Means within a column followed by different letters are significantly different at the mentioned probability.

It is reported that dry matter content of plants is most likely the best criterion of the degree of hardening in relation to a transition from in vitro to ex vitro conditions (Lakso *et al.*, 1986; Kozai *et al.*, 1988; Fari *et al.*, 1987). Similarly dry matter content was important in the transplantability of tree seedlings from the greenhouse to the field (Tolley *et al.*,1984). Kozai *et al.* (1988) reported that their in vitro plantlets acclimatized well based upon their enhanced growth in terms of dry weights. Dhawan and Bhojwani (1987) observed starch grain accumulation in palisade cells which preceded development of more normal mesophyll cells and acclimatization of plantlets. Leaf thickness has also been associated with acclimatization of plantlets in several reports (Wetzstein and Sommer, 1982; Donnelly and Vidaver, 1984; Smith *et al.*, 1990).

At 40 days from start of culture (10 days after transfer), transplant shock was clearly evident as seen in plant growth. This was more apparent in B-cap plantlets (Table 5.3). SPC plantlets maintained higher shoot fresh (1.25x) and dry weights for shoot, root and total dry weights (1.4x) as well as leaf area (1.27x). The difference stemmed mainly from the high leaf wilting and injury that took place with B-cap plantlets. B-cap plantlets had an injury score of 1.78 compared to SPC 1.27 upon transfer to ex vitro conditions (Table 5.3). SPC plantlets were more stress resistant although curling of the leaf edges took place which in some cases was irreversible. B-cap plantlets, however, showed curling and necrosis of whole leaves or large parts of them (Fig. 5.10). By day 50 (20 days after transplanting) B-cap plantlet growth appeared to recover as it had similar fresh weights, leaf areas and stem length to SPC plantlets but less dry weights and higher leaf damage.

Figure 5.10. In vitro tobacco plantlets from plastic B-cap (left) and semipermeable SP-cap (right) covered tissue culture vessels after 24 hours of transfer to the greenhouse, showing wilting and injury of leaves in plantlets from B-cap treatment as compared to those from SPC treatment.

However, by the sixtieth day of culture (30 days after transfer) B-cap plantlets seemed to completely recover their growth and exceeded that of SPC plantlets. They had significantly larger leaf number, stem length (Table 5.3) and relative growth rates (Table 5.5, Fig. 5.11). However, the dry weight/fresh weight and LAR (Table 5.4) did not differ. Plantlets from SPC treatments conserved their growth in terms of expansion and elongation and accumulated dry matter in a more compact morphology. This characteristic is typical of water stress resistant plants (Cutter, 1978; Manning *et al.*, 1977).

Since well-established root systems are critical for water and nutrient uptake of newly transplanted plantlets, a high root:shoot ratio may be advantageous in hardening of IVP's (Lakso *et al.*, 1986; Tolley *et al.*, 1984). Plantlets from SPC treatments had higher root:shoot ratio at 10, 20 and 30 days after transfer to the greenhouse (Table 5.4). Although these ratio may have been offset by the leaf damage on B-cap plantlets, a well developed root system is advantageous in providing water uptake and nutrients for growth necessary in hardening.

The relative growth rate (RGR) and net assimilation rate (NAR) in Table 5.5 carry the same trend as in Table 5.4 where RGR on a dry weight basis and NAR were enhanced for SPC plantlets while in vitro. A drop in the RGR is apparent after transfer to the greenhouse (Fig. 5.11) with recovery by day 50 and 60 especially for B-cap plantlets.

SPC plantlets, therefore, had better growth and less injury under transplanting stress. The increased dry weights in SPC leaves is indicative of fortified leaf structure. Increased LAR indicates accumulation of sugars and

Table 5.5. Relative growth rate (RGR) and net assimilation rate (NAR) of plantlets from vessels covered with B-cap and SP-cap, in vitro and after transfer to the Greenhouse (G.H).

		RGR[1] ($g\ g^{-1}$ per day^{-1})				NAR[2]
Period	Treatment	Leaf	Shoot	Root	Total	(g/LA per m^2 per day)
20-30 in vitro	B-cap	0.038 a	0.039 b	0.068 a	0.040 a	.097 b
	SPC	0.061 a	0.067 a	0.042 a	0.067 a	.208 a
	P-value	0.0594	0.0342	0.1958	0.0503	0.0123
30-40 in G.H.	B-cap	0.0311 a	0.034 a	0.021 a	0.025 a	0.07 a
	SP-cap	0.0350 a	0.040 a	0.011 a	0.034 a	0.12 a
	P-value	0.7631	0.6657	0.1451	0.5088	0.2468
40-50 in G.H	B-cap	0.079 a	0.085 a	0.098 a	0.087 a	0.26 a
	SP-cap	0.071 a	0.073 a	0.071 a	0.073 a	0.24 a
	P-value	0.5151	0.3846	0.0676	0.2991	0.6701
50-60 in G.H.	B-cap	0.092 a	0.102 a	0.103 a	0.102 a	.36 a
	SP-cap	0.058 b	0.071 b	0.068 b	0.070 b	.25 b
	P-value	0.0066	0.0086	0.0303	0.0090	.0075

(1) RGR = $[In(w_2)-In(W_1)]/(t_2-t_1)$, where W_1 and W_2 denote the dry weight at day t_1 and day t_2, respectively.

(2) NAR = $[(W_2-W_1)/(t_2-t_1)] \times [ln(A_2)-ln(A_1)]/(a_2-a_1)$, where W_1 and W_2; A_1 and A_2, denote the dry weight and leaf area at day t_1 and t_2, respectively.

* means followed by the same letter are not significantly different at the indicated probability.

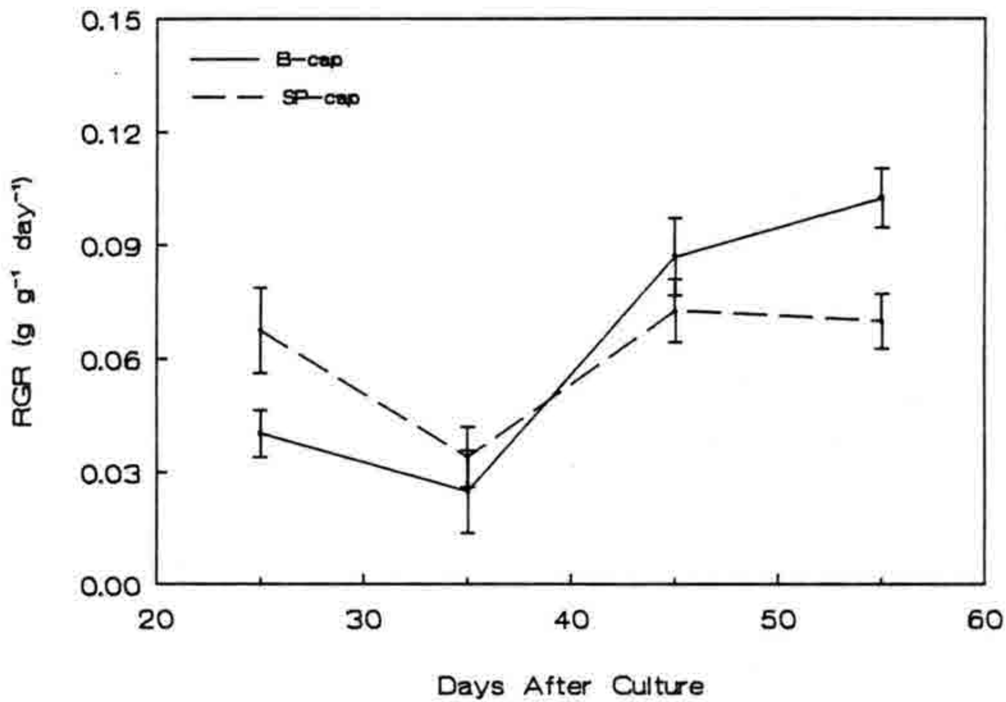

Figure 5.11. Relative growth rate (RGR; g g^{-1} day^{-1}) on total dry weight basis of tobacco plantlets grown in vitro in vessels covered with B-cap and SP-cap closures and followed in the greenhouse after transfer. Vertical bars indicate $\pm$ Standard Error.

starches. Although we cannot conclude the occurrence osmotic adjustment in our plantlets Increased dry weights were reported for plants grown under moderately low Ψ_{ω} and were attributed to accumulation of organic assimilates for osmotic adjustment (Janes, 1966). Pospisilova *et al.* (1988) reported osmotic adjustment in MS medium in plant cultures of tobacco. A Low leaf Ψ_{π} was also advantageous in promoting water uptake at time of transfer due to a large gradient in Ψ_{ω} between the plant and soil (Pospisilova *et al.*, 1988).

The enhanced dry weight in SPC plantlets could be due to better assimilation as related to better gas exchange and higher CO_2 concentration effected by the gas permeable SPC closure as compared to B-caps. In another part of this study, a higher CO_2 concentration was found in vessels covered with SPCs (Chapter IV). The B-cap plantlets, therefore, have lower dry weight accumulation due to limited CO_2 concentrations in in vitro cultures. However, plantlets photosynthesized normally under abundant CO_2 ex vitro and assumed autotrophy by day 50 as evidenced from the recovering NAR. Similar findings were found by Desjardins *et al.* (1987) and Pospisilova *et al.* (1987) who found that plantlets had limited photosynthetic rates and reduced biomass in vitro but had normal photosynthesis under abundant CO_2.

In conclusion, plantlets grown in SPC covered vessels demonstrate some features of hardening against transplant water stress. We do not know whether acclimatization was induced by the reduction in RH caused by the SPC gas permeability and consequent active transpiration in plantlets or by the effect of medium dehydration on inducing water stress characteristics in the plantlets. We

have demonstrated a slightly reduced RH, reduced Ψ_w of the medium and the plantlets in culture vessels covered with SPC.

A degree of control over water loss was observed which may be due to the effect of low water status on stomatal apertures as well as the demonstrated high epicuticular wax content combined with some inherent features of development (anatomy, stomates or trichomes) as affected by the low atmospheric RH and low media Ψ_w. Turgor maintenance in SPC could have been due to osmotic adjustment which could be partially responsible for the hardening. The accumulation of dry weight due to better gas exchange could also have contributed to development of wax coverage and possibly more compacted anatomy (mosophyll cells) as demonstrated by the lower LAR which indicates thicker leaves. A study of the internal and surface anatomy of plantlets from SPC treatments may clarify some acclimatization features induced by SPC. However, although plantlets from SPC treatments demonstrate relatively normal growth and behavior, it is still not known exactly how it handled the low water status at the two extremes (atmosphere and medium) to maintain transpiration and water uptake. Research pertaining to environmental physiology of in vitro-propagated plantlets as related to their water relations and acclimatization requires further investigations.

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CHAPTER VI

SEMIPERMEABLE COVERS ENHANCE STOMATAL FUNCTION AND PHOTOSYNTHESIS OF IN VITRO CULTURED TOBACCO

Introduction

Lack of autotrophic growth in in vitro-propagated-plantlets (IVP) and poor water relations have been suggested as major factors in the vulnerability of tissue-culture plantlets to stress after transplanting (Grout and Aston, 1977; 1978; Wardle and Short, 1983; Donnelly and Vidaver, 1984; Desjardins *et al.*, 1987). The characteristic high humidity, high sucrose concentrations, low illumination and low gas exchange of standard tissue culture growth conditions were reported to cause inadequate control over water loss and poor development of the photosynthetic apparatus (Grout and Aston 1978; Brainerd and Fuchigami, 1981; Lee *et al.* 1985; Donnelly and Vidaver, 1984; Langford and Wainwright, 1987; Evers 1982). However, other work suggested that the low photosynthesis rates of IVP were due the direct limitation of in vitro conditions on photosynthtic activity and not changes in the photosynthetic apparatus (Pospisilova *et al.*, 1987; 1988; Fujiwara *et al.*, 1987; Solarova, 1989; Aoki and Oda, 1988; Kozai, 1991).

Carbon dioxide concentrations [CO_2] in the tightly closed culture vessels were below the compensation point during most of the photoperiod (Abbott and Belcher, 1981; Fujiwara *et al.*, 1987; Pospisilova *et al.*, 1987; Solarova, 1989).

Chlorophyllous plantlets have full photosynthetic capacity but their low photosynthesis rates in vitro were largely due to limiting CO_2 and light in the vessel (Lee *et al.* 1985; Pospisilova *et al.* 1988; Fujiwara *et al.* 1987; Solarova, 1989; Kozai, 1991). It was possible, therefore, to improve photosynthetic rates by enriching the tissue culture environment with CO_2 (Mousseau, 1986; Fujiwara *et al.*, 1987). These treatments however, necessitated adequate gas exchange between the tissue culture environment and the external atmosphere.

Different closures for tissue culture vessels resulted in different levels of gas exchange in the culture vessel (Kozai *et* al., 1986). A gas permeable closure (semipermeable closure; SPC) made of transparent polymethylpentene film attached with microporous polypropylene film (giving 5.6 vessel air changes per hour), was devised to improve gas exchange and photon flux incidence (Kozai *et al.*, 1988a; 1988b). Plantlets grown in vessels covered with SPC under high CO_2 and high photosynthetic photon flux density (PPFD) had significantly higher growth rates compared to those grown under aluminum foil covers and low [CO_2] and light levels (Kozai *et al.*, 1988a; 1988b, 1990a; 1990b).

Based on the above information, a system was devised whereby plants were grown autotrophically in vitro using gas permeable closures, high [CO_2], high PPFD, and elimination of sucrose from the media (Kozai *et al.*, 1987; Kozai, 1988; Kozai *et al.*, 1988a; Hayashi *et al.*, 1988; Kozai *et al.*, 1990a; 1990b). This system was reported to produce vigorous plantlets, increase dry weights and enhance acclimatization in several plant species: potato (*Solanum tuberosum*; Kozai *et al.*, 1988a), carnation (*Dianthus caryophyllus*; Hayashi *et al.*, 1988; Kozai *et al.*, 1988b),

Rosa hybrida Hort. (Kozai *et al.*, 1990a) and *Cymbidium* (Kozai *et al.*, 1990b). The system was reported to improve acclimatization (Kozai, 1988; Hayashi *et al.*, 1988) although little information on specific aspects of plantlet acclimatization were given (Desjardins et al., 1988).

One of the major characteristics of acclimatization of IVPs involves restoration of stomatal functioning (Fuchigami *et al.*, 1981). Leaf conductance (g_s) is known to be high in IVPs, and is thought to be responsible for the excessive water losses which lead to desiccation of plantlets upon transplanting (Fuchigami *et al.*, 1981; Sutter, 1988).

Stomates respond directly to changes in atmospheric humidity, independently of bulk leaf water status and photosynthetic rates (Jarvis and Morison, 1981; Hall and Kaufmann, 1975a). This humidity-induced opening and closing of stomates is the result of gradients in turgor between guard cells and epidermal cells (Lange et al., 1971). Induction of this feature in IVPs should reduce transpiration and avoid stress (Jarvis and Morison, 1981).

Stomatal responses to CO_2 and light were reported to be independent of photosynthesis although a correlation exists (Jarvis and Morison, 1981). Photosynthesis is not affected by RH other than through effect of RH on stomatal conductance. Reduced stomatal conductance due to reduced ambient vapor pressure is the prime cause of humidity induced reductions in assimilation rate although the capacity for ribulose bisphosphate regeneration can be reduced by high transpiration rates (Farquhar and Sharkey, 1982). Under water stress conditions the responses of g_s to plant water status may also affect photosynthesis. Direct

effects of plant water status on photosynthesitic metabolism may also be realized through reduction in mesophyll photosynthetic capacity (Farquhar and Sharkey, 1982). Pre-exposure of plants to mild water stresses has been reported to acclimate the photosynthetic system to more severe water stress as well as reduce the threshold water potential at which stomates close (Jarvis and Morrison, 1981; Berkowitz, 1987; Downton, 1983).

The objective of this study was to explore the influence of the semipermeable cap on acclimatization of tobacco plantlets through examining its effect on plantlet responses to humidity. Carbon dioxide exchange rates (CER), conductance (g_s) and transpiration rates (E) were measured in response to reducing RH, different [CO_2] and darkness. This would be indicative of photosynthetic activity of plantlets and their stomatal responses to stomatal stimuli as affected by the SPC treatments.

Materials and Methods

Plant Culture. Tobacco 'Wisconsin 38' plantlets were grown in vitro from single nodal sections cultured on 0.6% agar-solidified Murashige and Skoog medium (Murashige and Skoog, 1962) with 3% sucrose and no hormones in 200 ml baby food jars. Two kinds of closures were used: B-caps (Magenta Corp., Chicago) and the polypropylene semipermeable caps (SPC; suncap closure; Sigma Chemical Company, Missouri). The SPC were autoclaved separately with Kimwipe tissues between the sheets and then used to close the sterilized culture jars aseptically. The cultures were grown on shelves in a culture room where PPFD at the plantlet level was 80-90 μmoles m^{-2} s^{-1} of continuous light provided by cool fluorescent

lamps with temperature of 25 $\pm$ 3°C and room relative humidity (RH) between 20% and 40%.

Greenhouse (GH) plants were started from seeds germinated in clay pots (6 inch) on 2 peat : 1 vermiculite : 2 perlite (by volume) in a growth chamber (Perceival, Boone, Iowa) at 25°C and 8/16 dark/photoperiod. The seedlings were then transferred to the greenhouse and fertilized with Peters 20N-10P-20K. Temperatures and RH in the greenhouse ranged between 20 to 27°C and 20% to 90% respectively. During most of the growth period RH ranged between 50 and 70%. PPFD levels ranged between 300 and 800 μmoles m^{-2} s^{-1}.

Leaf Gas Exchange. Single leaf gas exchange measurements were performed on the youngest, fully expanded leaf using the ADC steady state gas-exchange system (Analytical Development Co. Hoddesdon, England) with the model PLC(N) leaf chamber, LCA2 infrared gas analyzer, and ASU(MF) air supply unit. The measurements were made in a walk-in humidity-controlled growth chamber (M-11 Walk-in series, Environmental Growth Chambers, Chagrin Falls, Ohio). The [CO_2] in the chamber was maintained near ambient level by exhausting human exhalation through a mouth piece and tubing outside the chamber. Approximately 30 seconds was required to obtain a steady-state reading from a leaf. Air for the measurements was provided from bottled reconstituted and compressed air containing 460 to 480 μl L^{-1} CO_2 in air. To avoid desiccation of the leaf in the cuvette, air in the ADC system was humidified to about 38 to 40% RH (measured by the capacitance sensor at the cuvette) by bubbling it through water. Leaf conductance to water vapor, transpiration and CO_2 exchange rates were calculated

from equations [3] and [7] of Farquhar and Sharkey (1982). Temperature difference between the air inside the cuvette and the leaf was estimated by an energy balance equation developed for ADC as explained in Morgan *et al.* (1990). PPFD at plant height was 280 to 300 μmol m^{-2} s^{-1} supplied by H.I.D lighting. Mass flow was set at 200 ml min^{-1}.

I. Experiment 1. Gas exchange responses to humidity

Plantlets in culture were brought into the walk-in humidity chamber on the day of the measurement and allowed to acclimate for about one hour before measurements started. One plantlet at a time was taken out of the culture vessel and the leaf gas exchange was measured immediately (0 minutes), and measured again after 15, 30 and 60 minutes of exposure to the treatment of relative humidity. Four humidity treatments; 90%, 70%, 50%, and 30% were used in the walk-in chamber. One humidity level treatment per day was applied to a new set of plantlets each day. After gas exchange measurements, the portion of the leaf in the cuvette was cut and its leaf area determined using a leaf area meter (Model LI-3000A, LI-COR, Ltd.,Lincoln, Nebraska) in order to express gas exchange measurement on leaf area basis.

Statistics. The experiment was designed as a completely randomized, 4x3 factorial composed of 4 levels of humidity and 3 plant types: two grown in tissue culture with two different cap closures, SPC and B-caps, and the greenhouse seedlings. Four times of measurements at 0, 15, 30, and 60 minutes after exposure to the treatment humidity level were taken as repeated measures. Four to ten replicates for each combination were used. Multivariate analysis of variance

(MANOVA) for repeated measures was performed. The treatment means were separated for each humidity and time level as univariate analysis of variance using Student-Neuman-Keul's (SNK) method at the 5% level of probability. The effect of humidity was not analysed since due to the necessity of the experiment, one humidity level was applied per day per new set of plants which confounded humidity effect by the effect of the day. The ADC system presented another limitation for the experiment since the ADC does not operate well at RH level above 80%. Thus conductance of the control plantlets could not be determined at time 0 since were considered missing values since the cuvette humidity was over 80% RH.

II. Experiment 2. Gas exchange responses to [CO_2] and darkness

A second group of tobacco plantlets and seedlings were grown in culture and greenhouse under the same conditions and vessel closure treatments. The plantlets and seedlings were brought to the walk-in growth chamber and maintained at 85-90% RH for about one hour. Measurements of CER, gs and E were performed on the most recently fully expanded leaf as [CO_2] decreased (80 μl L^{-1}) and increased (600 μl L^{-1}) from ambient concentrations at 280 μmol m^{-2} s^{-1}, and also after one hour of darkness.

The experiment was designed as a completely randomized design with the gas exchange measurements analyzed as repeated measurements in the multivariate form (SAS, 1985). The means of the treatments for each variate were separated by SNK mean separation at the 5% level of probability.

III. <u>Experiment 3.</u> Chlorophyll content

Tobacco plantlets treated with the B-Cap and SPC and grown as explained above were assayed for their chlorophyll a and b according to Inskeep and Bloom (1985). The experiment was designed as a completely randomized design. The data were analyzed by one way analysis of variance and the treatment means were separated by SNK at the 5% level of significance.

IV. <u>Experiment 4.</u> CO_2 concentration in the culture vessels

Carbon dioxide concentration of the culture vessel gas phase was measured twice a day and after a period of dark on a separate set of plantlets each time. Treatments were B-Cap and SPC. A 5ml gas volume was sampled from the culture vessel using a hypodermic needle and injected into the stream of a Nitrogen gas carrier passing through a zeroed Infra Red Gas Analyzer (Model 865; Beckman Instruments, Inc. Fullerton, California). The data were analyzed as a one way analysis of variance for each set of measurements. The means were separated by SNK at the 5% level of probability.

Results and Discussion

I. <u>Experiment 1.</u>

Interactions between the effect of humidity and culture condition treatments (vessel closure) were significant in most cases (Table 6.1). Therefore, photosynthesis (Fig. 6.1), leaf conductance (Fig. 6.2) and transpiration (Fig. 6.3) measurements are presented separately for each treatment at each measurement time.

Table 6.1. Probabilities for single leaf gas exchange of B-Cap, SP-Cap plantlets and greenhouse plantlets at 0, 15, 30 and 60 minutes time of measurement.

Source		CER[1]0	CER15	CER30	CER60
humidity	(RH)	.8093	.0052	.0001	.0001
treatment	(trt)	.0001	.0001	.0001	.0001
RH * trt		.1075	.0013	.0001	.0005
		g_s[1]0	g_s15	g_s30	g_s60
humidity		.0032	.0002	.0001	.0001
treatment		.0001	.0002	.0928	.3935
RH * trt		.0538	.0527	.0074	.2630
		E[1]0	E15	E30	E60
humidity		.0001	.0001	.0001	.0001
treatment		.0001	.0001	.0006	.0341
RH * trt		.0001	.0918	.0260	.1551

[1] CER = CO_2 exchange rate; g_s = stomatal conductance to water vapor; E = transpiration rate; 0, 15, 30 and 60 are the times in minutes after removal from culture and at which measurements were done.

Fig 6.1. CO_2 exchange rate (CER) of tobacco plantlets and greenhouse plants (GH) as affected by time of exposure to 30% RH (a), 50% RH (b), 70% RH (c), and 90% RH (d). The response to humidity at each time of exposure is shown in (e) for 0 minutes, (f) for 15 minutes, (g) for 30 minutes and (h) for 60 minutes. Vertical bars represent $\pm$ standard error.

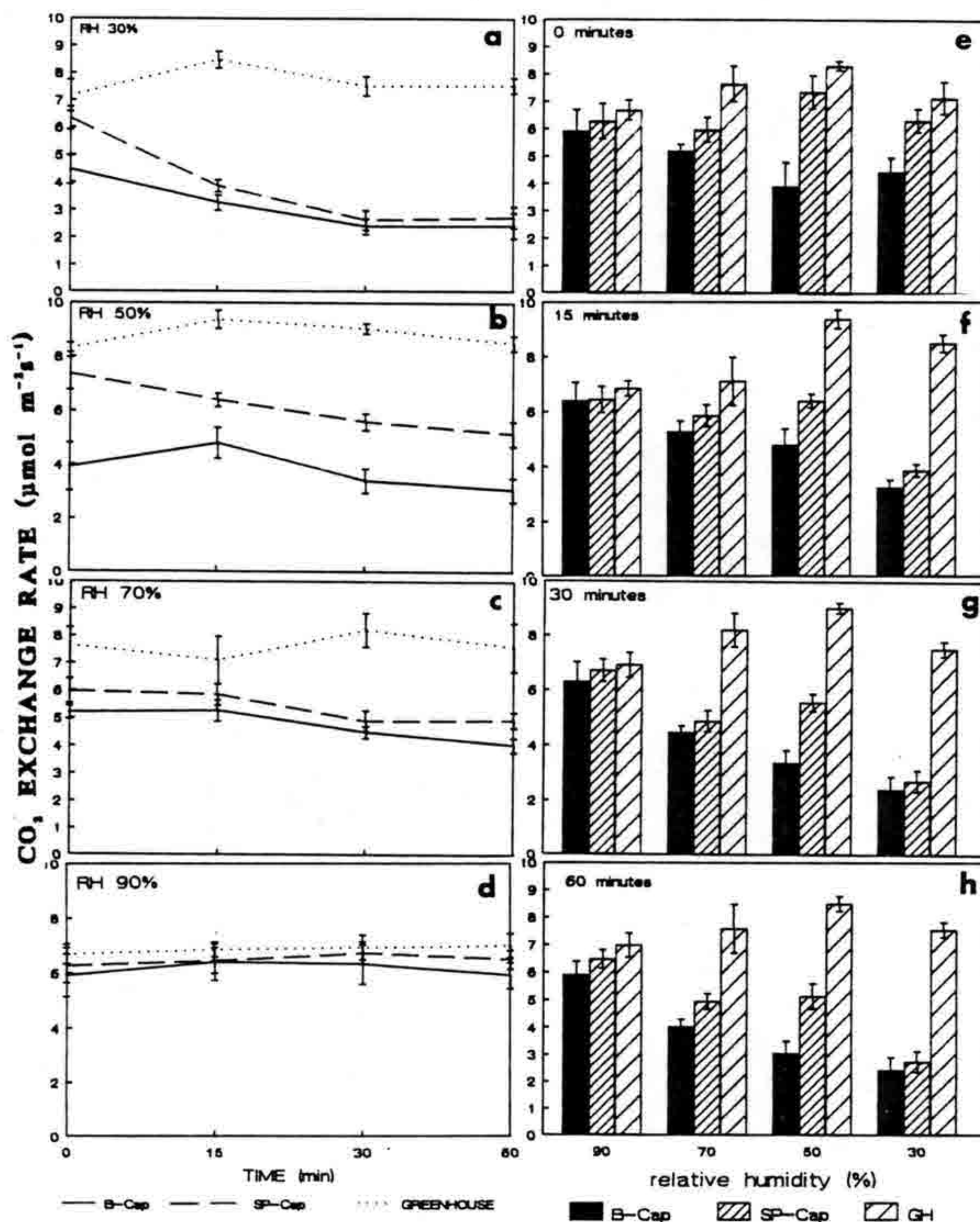

RH 30%
a
RH 50%
b
RH 70%
c
RH 90%
d
0 minutes
e
15 minutes
f
30 minutes
g
60 minutes
h
CO_2 EXCHANGE RATE (μmol $m^{-2}s^{-1}$)
TIME (min)
relative humidity (%)
B-Cap
SP-Cap
GREENHOUSE
GH

Fig. 6.2. Stomatal conductance (g_s) of tobacco plantlets and greenhouse (GH) plants as affected by time of exposure to 30% RH (a), 50% RH (b), 70% RH (c), and 90% RH (d). The response to humidity at each time of exposure is shown in (e) for 0 minutes, (f) for 15 minutes, (g) for 30 minutes and (h) for 60 minutes. Bars are standard error of the mean.

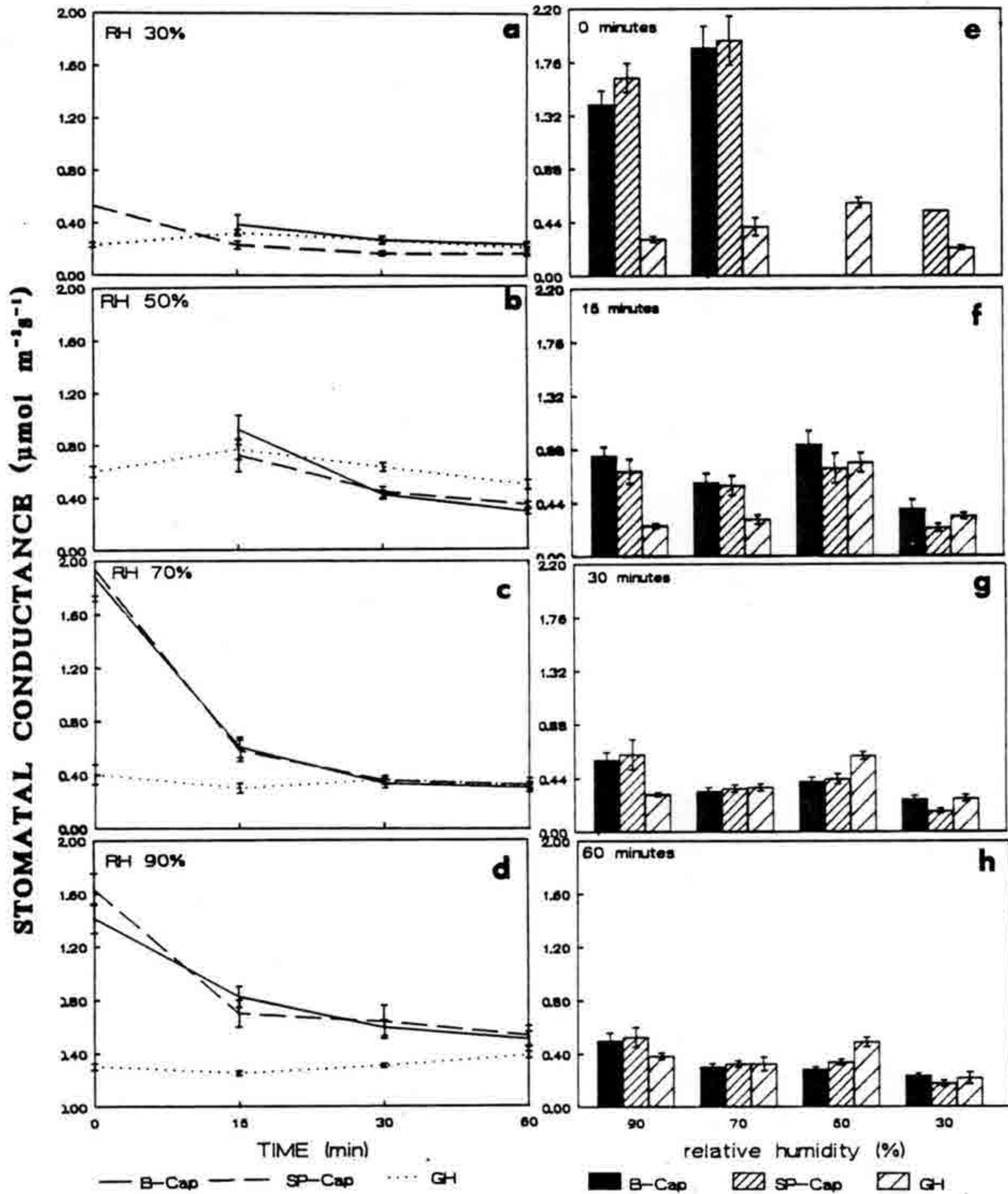
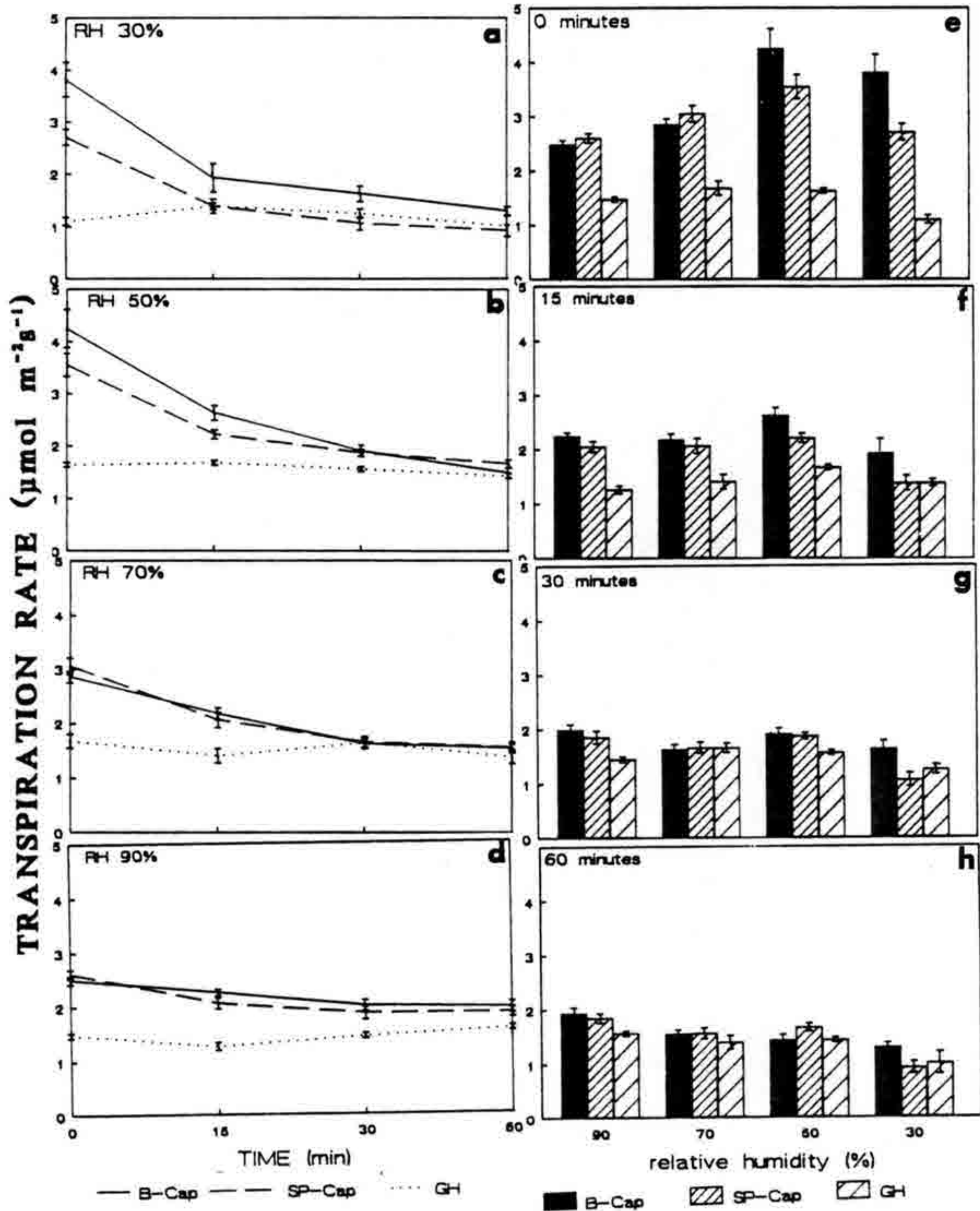

STOMATAL CONDUCTANCE (µmol m⁻²s⁻¹)
RH 30%
a
RH 50%
b
RH 70%
c
RH 90%
d
0 minutes
e
15 minutes
f
30 minutes
g
60 minutes
h
TIME (min)
relative humidity (%)
B-Cap
SP-Cap
GH
B-Cap
SP-Cap
GH

Fig. 6.3. Transpiration rate of tobacco plantlets and greenhouse (GH) plants as affected by time of exposure to 30% RH (a), 50% RH (b), 70% RH (c), and 90% RH (d). The response to humidity at each time of exposure is shown in (e) for 0 minutes, (f) for 15 minutes, (g) for 30 minutes and (h) for 60 minutes. Bars are standard error of the mean at 5% level of significance.


RH 30%
a
RH 50%
b
RH 70%
c
RH 90%
d
TRANSPIRATION RATE (µmol m⁻²s⁻¹)
TIME (min)
— B-Cap
— — SP-Cap
···· GH
0 minutes
e
15 minutes
f
30 minutes
g
60 minutes
h
relative humidity (%)
B-Cap
SP-Cap
GH

Manova indicated highly significant ($P < 0.01$) effects of time, in addition to two and three-way interactions of time with effects of humidity and treatment onCER, g_s and E, with one exception; the three-way interaction of timeXhumiditXtreatment was not significant for g_s. The following discussion elaborates on many of these interactions.

Carbon dioxide exchange rates were greater at all measurement times for the greenhouse (GH) plants at 70%, 50% and 30% RH (Fig. 6.1). Carbon dioxide exchange rates for plantlets covered with SPC exceeded those with B-caps, at 50% RH, and during the first 15 minutes at 30% RH (Fig. 6.1a,b,c,d).

These results show that exposure to RH below 90% appeared to impair photosynthesis in plantlets, and that the degree of impairment was related to culture vessel closures used. This implies an effect of water relations of the plantlets on photosynthesis of IVPs. Photosynthesis of SPC-grown plantlets appeared initially to be less sensitive to the lower humidities, although all plantlets reached similar CER after 1 hour exposure to 30% RH (Fig. 6.1a). Similar CER in all treatments at 90% RH (Fig. 6.1d) support previous findings (Fujiwara *et al.*, 1987; Pospisilova *et al.*, 1987; Solarova, 1989; Kozai, 1991) which suggest that photosynthetic machinery of plantlets is not necessarily impaired. Our results clearly indicate that low photosynthesis of in vitro plantlets is related more to their vulnerability to water stress as imposed by decreasing RH.

The degree of humidity responses of the treatments can be visualized easier in Fig. 6.1e-h. Only the in vitro plantlets responded to reduced humidity by reducing CER, which again shows the vulnerability of plantlet photosynthesis to

humidity-induced water stress. B-cap plantlets' CER was reduced more than those of SPC plantlets in response to RH. Greenhouse plant maintained a steady state of photosynthesis. These results agree with Fraquhar and Sharkey (1982) and Wong et al. (1979) who reported reduced assimilation rates with rapid imposition of water stress due to transpiration.

At time zero where comparisons between the IVPs and greenhouse-grown plants could be made (see materials and methods), IVPs had significantly greater g_s compared to greenhouse-grown plants (Fig. 6.2). These initially high g_s of the IVPs resulted in high transpiration rates (Fig. 6.3), and presumably, greater desiccation potential relative to the GH-grown plants. Although no direct measurements of IVP (or plant) water status were made, the reductions in g_s and E with time for the IVPs (Fig. 6.2 and 6.3) provide indirect evidence of stomatal closure in response to humidity or plant water stress. Tissue desiccation due to the initially high E of the IVPs may account for the humidity-induced photosynthetic inhibition observed in these plantlets (Fig. 6.1). The slightly lower but significant g_s (and consequent lower E [Fig. 6.3]) determined in SPC plantlets at lower humidities in the first 15 minutes of the 60-minutes exposure period (Fig. 6.2) similarly suggests initially less desiccation potential for SPC plantlets, and helps explain their greater photosynthetic resistance to low RH compared to B-cap grown plantlets (Fig. 6.1).

We propose three possibilities 1) SPC induced adaptation of IVP stomates thus mitigating the humidity induced desiccation and consequent injury to the photosynthetic apparatus caused by exposure to low humidity (stress avoidance), 2)

SPC induced adaptation of the photosynthetic system to drought due to pre-exposure to water stress (tolerance), and 3) the photosynthetic parameters (CO_2 compensation point) changed due to the cap treatment such that low internal CO_2 concentrations caused by stomatal closure in response to humidity limited CER rates to a less degree in SPC than B-cap vessel grown plantlets (tolerance). Photosynthesis of plants from the GH were least limited by stomatal closure indicating lower compensation point. This agrees with previous work which reported increased CO_2 compensation point of IVPs (Pospisilova *et al.*, 1987; 1988) and the improvement of this parameter by enhancing photosynthetic activity (Kozai *et al.*, 1990b; Kozai, 1991).

These results show that plantlets respond to exposure to ex vitro humidity conditions by reducing the stomatal conductance and transpiration but the response is neither complete nor fast enough to avoid water stress upon transfer especially at low RH of 30%. The slow stomatal response of IVPs was reported in another work in our laboratory (Safadi *et al.*, 1990) and in Shackel *et al.* (1990). The inefficiency in avoiding water stress shows by the adverse effect on the photosynthetic rates at low humidities of 30 and 50% RH.

SPC could have improved plantlet CER under low RH by avoiding water stress through improved stomatal functioning and epicuticular wax coverage. In another part of our study, we have demonstrated significantly higher epicuticular wax coverage and dry weight accumulation in SPC plantlets (Safadi and Hughes, 1991) but we did not study the stomatal apertures other than what was indicated by the reduced transpiration in this experiment. On the other hand, SPC could

have caused some kind of acclimation to the photosynthetic system of the plantlets by pre-exposing plantlets to some degree of water stress in vitro. Preacclimatization to water stress by osmotic adjustments was reported to protect the photosynthetic apparatus against water stress-induced photoinhibition (Downton 1983) . Similarly, chloroplast pre-conditioning was reported by Berkowitz (1987). However, this kind of acclimation may require high water stress to cause osmotic adjustment of the plant or chloroplast. In another part of our study, we have shown that SPC plantlet leaves and their media had lower water potential than that of B-Cap plantlets and the humidity in the jars was slightly lower (Safadi and Hughes, 1991). This may have shifted the optimum osmotic potential for photosynthesis to a lower value as observed by Berkowitz (1987) with preacclimatized spinach (*Spinacia oleracia* L. cv. 'Winter Bloomsdale'). This conclusion, however, cannot be made unless the relationship between the osmotic potential of SPC plantlets and their photosynthetic responses are studied. In another scenario, the improved gas exchange in SPC may have enhanced photosynthesis in vitro and thus have improved stomatal functioning. A correlation between photosynthesis and stomatal conductance has been reported (Wong *et al.* 1979). Enhanced leaf gas exchange due to SPC may also decrease the CO_2 compensation point. This effect was reported for IVPs with enhanced photoautotrophy (Kozai, 1991).

Kozai and coworkers (Kozai, 1991) have assumed that their system which enhances photoaututrophy in IVPs would improve acclimatization by improving growth and dry weights. This study shows that SPC caused some stomatal closure which could have protected the plants to a limited degree from transplanting

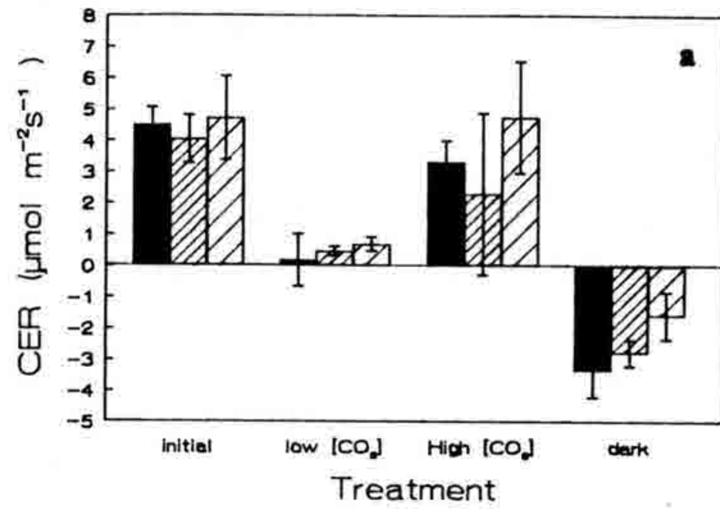
desiccation effect on photosynthesis. The study also shows that slow stomatal closure take place in non-treated plants. SPC effect on gas exchange improved the response of plantlets to water stress by reducing conductance and transpiration, and possibly by improving the adaptation of the photosynthetic system to water stress.

II. Experiment 2.

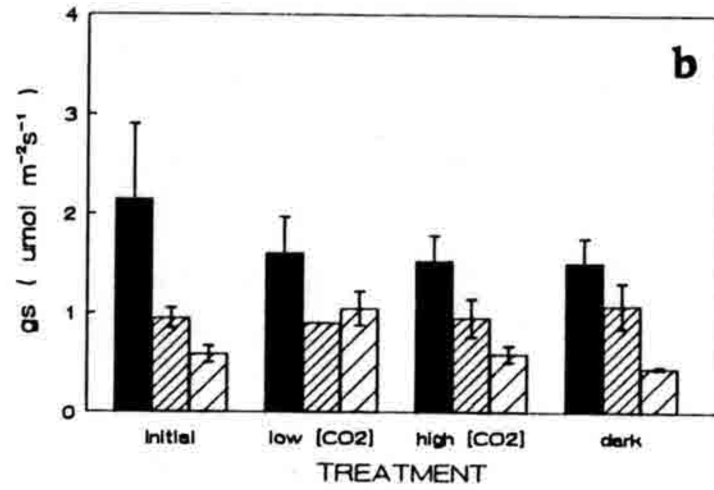
Photosynthetic rates and stomatal function were measured at low CO_2 concentrations, high CO_2 concentrations and darkness under nonlimiting RH of 85-90%. The rate of photosynthesis showed no significant differences among B-Cap, SPC plantlets and GH plantlets under all treatments of low CO_2, high CO_2 and darkness (Figure 6.4a). This result is in accordance with the results of the previous experiment where the three treatments did not differ in photosynthetic rates at the non-stressing humidity level of 90% RH. This further supports the conclusion that plantlets have a normal photosynthetic system and full photosynthetic capacity under non-water stressing regimes and as also reported by Pospisilova *et al.* (1988), Fujiwara *et al.* (1987) and Kozai (1991). Plantlets from B-cap treatments had more negative carbon balance in the dark than B-cap and greenhouse plants plantlets (not statistically different). This may be due to higher respiration rates of IVPs, as observed by Kozai and Iwanami (1988).

Due to the variability of the data for g_s we calculated a squareroot-transformation of g_s. Greenhouse plants responded to low [CO_2] by increasing their g_s (Fig. 6.4b) and E (Fig. 6.4c), and to high [CO_2] and darkness by reducing g_s and E (Fig 6.4a and 6.4c respectively). In comparison, g_s and E of SPC and B-Cap plantlets failed to respond to low and high CO_2 and darkness. Higher g_s and E

Fig. 6.4. **a)** Carbon dioxide exchange rate (CER), **b)** stomatal conductance (g_s) and **c)** transpiration rates (E) of in vitro tobacco plantlets and greenhouse plants as affected by stomatal function stimuli of low [CO_2] of 80 μl L^{-1}, high CO_2 660 μl L^{-1}, and darkness. Bars are standard error of the mean at 5% level of significance.

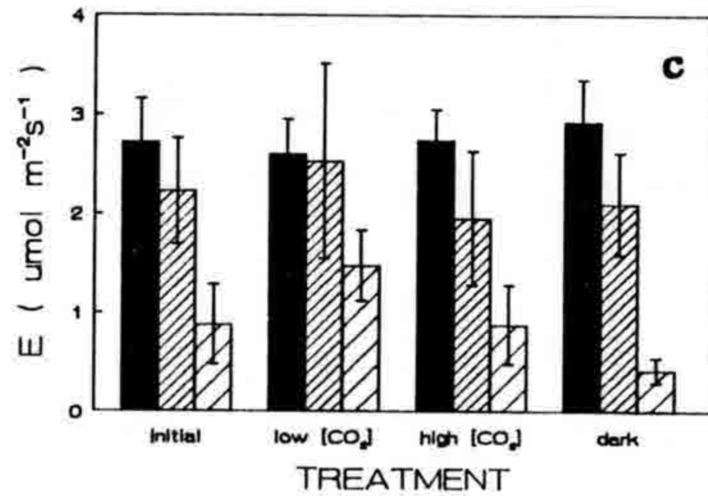


a
CER (μmol m-2s-1)
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
initial
low [CO2]
High [CO2]
dark
Treatment



b
gs (umol m-2s-1)
4
3
2
1
0
initial
low [CO2]
high [CO2]
dark
TREATMENT

B-Cap
SP-Cap
GH



c
E (umol m-2s-1)
4
3
2
1
0
initial
low [CO2]
high [CO2]
dark
TREATMENT

were observed for IVPs than those for GH plants under all conditions (Fig. 6.4b). Plantlets grown in vessels covered with SPC had slightly lower g_s and E than B-cap plants under all conditions, although it does not show statistical significance (P= 0.105 initial, 0.3217 at low [CO_2], 0.023 at high [CO_2], and 0.0091 at darkness; Fig. 6.4b). These results show an impairment of stomatal function in the B-Cap plantlets and to a lesser extent in the SPC plantlets as compared to the GH plants. Failure of stomates of IVPs to respond to known stomatal stimuli has been reported by several other researchers (Wardle and Short 1983; Brainerd and Fuchigami, 1982; Ziv *et al.*, 1987).

It is reported that at high humidity (90%), plants respond little to changes in [CO_2] (Hall and Kaufmann, 1975b; Jarvis and Morison, 1981). In this experiment (experiment 2) the stomates of IVPs remained open under high humidity inspite of the stomatal closure stimuli. However, SPC plantlets' g_s were always intermediate between GH and B-cap plants.

We propose that SPC caused some reduction in stomatal conductance and a small degree of restoration of stomatal function which may explain its contribution to water stress resistance in experiment one. The high humidity during the experiment or the relative low number of replicates may have limited the demonstration of more substantial response of SPC plantlets to stomatal stimuli.

III. Experiment 3.

Leaf chlorophyll assays (Table 6.2) showed significantly higher chlorophyll contents for the SPC than the B-Cap plantlets. This may explain the

Table 6.2. Leaf chlorophyll (chl) content of the treated plantlets.

Treatment	mg cm^{-2}		
	Chl a	Chl b	Total Chl
B-Cap plantlets	1.5399 b[1] ($\pm$0.154)[2]	0.39325 b ($\pm$0.0404)	1.9327 b ($\pm$0.1946)
SPC plantlets	2.404 a ($\pm$0.0636)	0.63893 a ($\pm$0.0193)	3.0427 a ($\pm$0.0819)

[1] means are separated using Student-Neuman-Keul's mean separation, means followed by the same letter are not significantly different at the 5% level of probability.

[2] Standard error of the mean.

higher photosynthetic rates encountered in some cases in SPC plantlets in experiment one. SPC plantlets therefore may have had more photosynthetic laminae. Chlorophyll contents for plantlets grown under improved gas exchange systems have not been reported. However, higher amounts of chlorophyll were associated with acclimatized plantlets (Ziv *et al.*, 1983; Smith *et al.*, 1990). On the other hand, chlorophyll content has not been clearly associated with increased photosynthetic capacity (Bjorkman and Holmgren, 19631 Langford and Wainwright, 1987). Chlorophyll increase may simply be a result of the overall enhanced assimilation rates in vitro and accumulation of metabolites due to SPC.

IV. Experiment 4.

[CO_2] in the gaseous phase of the culture vessels was always higher in the SPC covered jars than in those covered with B-Caps after a period of light

in the morning or the afternoon (Table 6.3). The CO_2 level in the jars, however, was always lower than that in the atmosphere, again, indicating the limitation of CO_2 for photosynthesis in in vitro conditions. After a period of darkness, CO_2 was very high in both types of capped jars but was significantly higher for the jars with B-Caps. This finding was reported by several other workers (Abbott and Belcher, 1981; Kozai and Sekimoto, 1988; Kozai *et al.*, 1990a) who indicated the limitation of the closures for gas exchange. Enhancement of gas exchange was provided by the SPC coover as it was designed for (Kozai *et al.* 1990b) but not to levels comparable to ambient [CO_2]. The need for CO_2 enrichment to keep CO_2 levels in the jar near the ambient atmospheric or higher levels is implied.

In conclusion SPC have improved photosynthesis of in vitro plantlets and resulted in some reduction in stomatal conductance and transpiration under desiccating RH. The reduction in g_s may have avoided some influence of water stress on photosynthesis. SPC also may have improved photosynthetic parameters and caused acclimation of the photosynthetic system to water stress. A limited improvement in the response of stomates to stomatal closure stimuli, [CO_2] and darkness, was provided by SPC. SPC improved chlorophyll content of plantlets which may be related to improving photosynthetic machinery. SPC also increased CO_2 levels in the culture vessels for photosynthesis of IVPs. This may be associated with improved photosynthetic parameters. These inductions by SPC are vital for the acclimatization of tissue culture plantlets upon transfer to ex vitro conditions. However, to study the effect of improved photosynthesis and photoautotrophy on acclimatization of IVPs, a substantial improvement in CER

Table 6.3. Mean CO_2 concentrations ($\mu l\ L^{-1}$) of the gaseous phase in the growth vessels containing a 2-week-old tobacco plantlet.

Time of the day	Treatment	$[CO_2]\ \mu l\ L^{-1}$	
morning after continuous light	B-Cap	91 ($\pm$9.4106)[2]	b[1]
	SPC	138 ($\pm$13.9902)	a
morning after 8 hours dark	B-Cap	1710 ($\pm$76.4735)	a
	SPC	1235 ($\pm$50.4679)	b
noon	B-Cap	152 ($\pm$17.857)	b
	SPC	218 ($\pm$23.098)	a
6:00 p.m.	B-Cap	98 ($\pm$4.2057)	a
	SPC	151 ($\pm$7.3742)	b

[1] means are separated using Student-Neuman-Keul's mean separation, means followed by the same letter are not significantly different at the 5% level of probability.
[2] Standard error of the mean.

should be induced. This can be done by enriching with CO_2 and light and eliminating sucrose, besides the use of semipermeable caps.

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