The Influence of *In Vitro* KCl Treatments on the Water Relations and Acclimatization of Tissue-Cultured Flame Azalea (*Rhododendron calendulaceum*)

by

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ABSTRACT

Propagation by tissue culture is effective for many woody ornamental plants, but propagules often become desiccated and die during the acclimatization period. This loss is due in part to stomata that fail to close in response to the reduced humidity outside of the culture environment. KCl was used in *in vitro* treatments to determine if an additional K supply would improve microshoot stomatal function and water status during acclimatization. The effects of the KCl treatments upon subsequent microshoot rooting and percent fresh weight gain were also evaluated.

In preliminary experiments, microshoots of the flame azalea (*Rhododendron calendulaceum*) were subcultured onto modified Woody Plant Medium amended with a wide range of KCl concentrations for various time periods. It was determined that microshoots did not grow well when cultured at *in vitro* KCl levels above 50 mM, so treatments were adjusted to 0, 30, and 60 mM KCl, with 9 days of *in vitro* exposure. After treatment, percent tissue K was determined by atomic absorption spectrophotometry and microshoot water potentials were measured by thermocouple psychrometry.

The capacity for the microshoots to resist desiccation after in vitro KCl treatment was determined by percent rooting and fresh weight gain after exposure to dehydration stress, and by gravimetric weight loss of microshoots placed in isopiestic tubes. In addition, microshoots from in vitro KCl treatments were evaluated for percent stomatal closure and water potential during a 38-day acclimatization period.

In vitro KCl treatments induced elevated tissue K levels in microshoots and reduced microshoot water potentials, but it could not be shown that these effects specifically enabled the microshoots to resist desiccation. Rooting and percent fresh weight gain were not affected by in vitro treatments, nor was gravimetric weight loss. Microshoot maturation, as a function of days out of culture, had the greatest effect upon increased stomatal function, which, coupled with the onset of rooting, improved microshoot water status.

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DEDICATION

For Dick, who is always there.

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CHAPTER ONE: REVIEW OF LITERATURE

Introduction

Plant tissue culture has received much attention in the last decade as a means of generating new plants in quantities unprecedented by any other asexual propagative method. In the United States alone, about 75 million plants yearly are commercially micropropagated by tissue culture, to provide foliage and flowering plants, woody ornamentals, and fruit crops (Einset, 1987).

For the nurseryman, the advantages of propagation by tissue culture include production of exponential numbers of disease-free plants that are uniform in quality and size, as well as year round employment for propagators (Einset, 1987). Inventories of cultivars that are in high demand can be built up quickly by micropropagation, which is advantageous if the cultivar happens to be difficult to propagate conventionally or if there has been a crop failure (Knuttel, 1989).

There are good reasons for growers of woody ornamentals to be excited about the potential of tissue culture. First, as mentioned, many desirable woody species that are difficult to propagate asexually have been micropropagated, such as almond, Prunus amygdalus Batsch (Rugini and Verma, 1982), mountain laurel, Kalmia latifolia (Lloyd and McCown, 1980), and several conifers (Cheng and Voqui, 1977). Second, the tissue culture of a sport or otherwise rare specimen reduces the risk of losing unique germplasm.

A single shoot tip may yield 10-20 individual cultures, which give a higher probability for survival than if that same shoot tip were to be used as a single cutting. Third, most fruit tree and ornamental cultivars are highly heterozygous, so that their seed progeny are not true-to-type (Bhojwani and Razdan, 1983). Micropropagation produces genetic clones of the parent plant, which permits the perpetuation of the unique characteristics of the cultivar.

Micropropagation of Native Species. Micropropagation may assist the commercial grower in producing native species on a large scale for inclusion in landscapes. Many indigenous plants are both attractive and undemanding with respect to fertilizer and water requirements. Concern for surface and ground water contamination by fertilizer leachate and future scarcity of good quality water will undoubtedly shift the focus on landscape materials to environmentally compatible native plants.

Flame azalea (Rhododendron calendulaceum [Michx.] Torr.) is a deciduous azalea native to the Appalachian Mountains, and produces orange to yellow flower trusses in late May. Commercial growers have not actively produced this plant for sale because of the difficulty in vegetatively propagating it. The seedlings are highly variable with respect to form and flower color, as well. Flame azalea has been successfully micropropagated, however (Blazich and Acedo, 1988), and this technique may provide a method for the plant to become more widely distributed. In the eastern United States, other deciduous azaleas, such as R. alabamense (Alabama Azalea), R. arborescens (Sweet Azalea), and R. atlanticum (Coast Azalea) would

contribute greatly to any landscape design, and probably could be micropropagated as easily as the flame azalea.

Stages of Micropropagation. Three stages of micropropagation by tissue culture have been defined by Murashige (1974). Stage I is the establishment of an aseptic culture of the plant material, during which the cellular physiology changes from an autotrophic (photosynthetic) to a heterotrophic (non-photosynthetic) state. In Stage II, the propagules undergo rapid clonal multiplication by axillary shoot elongation and proliferation, in response to exogenous growth regulators. Stage III, or acclimatization, involves preparing the microshoots for reestablishment in a soilesss medium, with a concomitant return to an autotrophic metabolism. During this last stage, the microshoots must also produce roots and acclimate to the generally higher light level and reduced humidity outside the culture vessel.

Problems Associated with Acclimatization. The high humidity inside the culture vessel promotes rapid shoot proliferation and growth, but also permits abnormal plant development (Ziv et al., 1983, 1987). Several physiological and morphological aberrations that arise during tissue culture hinder the acclimatization process, such as insufficient epicuticular wax deposition (Grout and Aston, 1977; Sutter and Langhans, 1982; Fuchigami, et al., 1981), reduced levels of chlorophyll a, O_2 evolution during noncyclic photophosphorylation (Hill reaction), and CO_2 fixation (George and Sherrington, 1984). Whereas tissue-cultured shoots do contain chlorophyll and have slight photosynthetic capacity, they do not fix adequate amounts of

CO_2 for maintenance and growth (Grout and Aston, 1978). Thus, in vitro cultures must be sustained by exogenous carbon in the medium (Haissig, 1974).

During the transition period from aseptic culture to the greenhouse, excessive desiccation of the microshoots can occur, caused in part by non-functioning stomata that fail to close in response to the stimulus of reduced humidity (Brainerd and Fuchigami, 1982; Wardle, et al., 1983a; Conner and Conner, 1984; Sutter, 1988). Stomata of cultured plants do not seem to have a closure mechanism, but apparently acquire one during acclimatization (Brainerd and Fuchigami, 1981).

Control of Relative Humidity. The greatest losses of tissue-cultured plants occur during acclimatization because of microshoot desiccation. Much effort has been directed toward the development of conditioning regimens that focus on gradual exposure to reduced humidity. These regimens include both ex vitro and in vitro manipulations of the cultured plant environment. Commercial nurseries employ fog tents and intermittent mist systems for the ex vitro conditioning of plantlets (George and Sherrington, 1984). The relative humidity in these systems is gradually reduced and the plantlets respond to this stimulus by producing functional stomata, as well as other normal physical features, from de novo growth (Brainerd and Fuchigami, 1981). From this point onward, the care of tissue-cultured stock is similar to that of seedlings and tip cuttings.

In vitro reduction of relative humidity has been used experimentally,

with some success. Ziv, et al. (1983) grew carnation microshoots on top of sterile anhydrous $CaSO_4$ in an attempt to induce epicuticular wax to form on the leaves. The desiccant caused the humidity in the culture vessels to drop from 96 to 92%, resulting in both a significant increase in shoots with normal leaf morphology, and improved survival rate during acclimatization, although with reduced shoot proliferation.

Relative humidity in a culture vessel also can be reduced by adding osmotically active reagents to the culture medium (Harris, et al., 1970). Addition of these reagents also has been shown to reduce the osmotic potential of the plant cell sap (Kimball, et al., 1975; Klenovska, 1976; Maretski, et al., 1972).

Components of Plant Water Potential. A loss of turgor, by desiccation, results in wilting that may be lethal, especially for microshoots. Plant water status may be determined by measurement of plant water potential. Total water potential consists of 2 components, turgor potential and osmotic potential. Turgor potential is the positive pressure of cell contents pressing the protoplast membrane against the cell wall. Osmotic potential has a negative pressure value because the presence of solutes such as vacuolar contents reduces the free energy state of the cell sap (Kramer, 1983; Chang, 1981). A reduction in total water potential (more negative value) may indicate either turgor loss or greater concentration of solutes in the cell sap, or a combination of both.

Reduction of plant water potential by solute accumulation may be

beneficial as a conditioning program for moisture stress. Eakes, et al. (1989) found that increasing the K fertilizer rate and exposing Salvia plants to sub-lethal dry-down cycles (moisture stress conditioning) resulted in active osmotic adjustment and increased cell turgor potentials. The Salvia leaves accumulated K, which likely decreased the osmotic potential within the cells. The cells then were able to retain water and maintain turgidity when subjected to dehydration stress.

Plant Water Status and K Nutrition. K is both osmotically active and an important nutritional requirement for cells grown in culture. K is considered to be the major ion responsible for maintenance of good plant water balance (Kramer, 1983; Mengel and Kirkby, 1987) and its absence in plant nutrition is characterized by a loss of plant turgor (Macrobbie, 1981; Mengel and Arnecke, 1982).

K is taken up in high rates by plant tissues (Brag, 1972; Dhindsa, et al., 1975) and once there, is very mobile (Mengel and Arnecke, 1982). In studies using RbCl for KCl, rubidium accumulated by chrysanthemum leaves developed in vitro was translocated to new growth during acclimatization (Wardle, et al., 1983b). Wardle, et. al. (1979) theorized that tissue-cultured plantlets act as storage organs for minerals which are subsequently utilized during the establishment of the plantlets in soil.

K seems to be important in normal cuticle development as well (Mengel and Arnecke, 1982), which is a contributing factor in proper water balance. Low K levels in tissue culture medium have been linked to vitrification

(Sutter, 1988), a condition which gives a water-soaked appearance to leaves, often followed by rapid necrosis and death of the culture.

K has long been linked to stomatal function. Stomatal aperture is directly related to the relative volume of guard cells, and is dependent upon turgor pressure. Turgor buildup and stomatal opening follow upon ion accumulation in guard cell vacuoles; K^+ is the primary cation and Cl^- and malate are the counterbalancing ions (Allaway, 1981). Studies with cauliflower plantlets showed that an uptake of water in cells and tissue was frequently a consequence of active K^+ uptake (Wardle, et al., 1979). Plantlets on low K treatment showed lower turgor potential than did plantlets on high K treatment. Mengel and Arnecke (1982) found the same correlation in Phaseolus; in young leaves K^+ was indespensible for obtaining optimum cell turgor, in turn required for cell expansion (Cleland, 1971).

Transpiration rate is affected by K nutrition levels because of the connection of K to stomatal aperture. Brag (1972) found that wheat grown with high K levels had a lower transpiration rate than did K-deficient wheat when correlated with root-shoot ratios, stomatal frequency, and stomatal aperture. Addition of KCl to K-deficient wheat plants reduced the transpiration rate by 50% in 2 hrs. This reduction in transpiration could be attributed to reduction in osmotic potential of mesophyll cells as well as stomatal aperture (Brag, 1972).

Thus, there is a twofold function of K that can be exploited for an in vitro acclimatization regimen; namely, high K levels in plant tissue are

available when functional stomates do develop from de novo growth, and high K accumulation in the plant may reduce overall microshoot water potential. This may enable the microshoots to adjust osmotically when subjected to dehydration stress during acclimatization. In addition, free water may flow into the plantlets in response to the water potential gradient between the more negative plantlet and the surrounding medium.

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CHAPTER TWO: THE INFLUENCE OF *IN VITRO* KCl TREATMENTS ON THE WATER RELATIONS AND ACCLIMATIZATION OF TISSUE-CULTURED FLAME AZALEA (*Rhododendron calendulaceum*)

INTRODUCTION

Plant tissue culture provides methods for fast, efficient propagation of a wide variety of plant species. There is particular interest in the micropropagation of woody ornamentals, and many desirable plants that are difficult to propagate by conventional vegetative methods have been successfully micropropagated (Lloyd and McCown, 1980).

For instance, flame azalea (*Rhododendron calendulaceum* [Michx.] Torr) is a native deciduous species admired for its orange flowers. It is not commercially grown to any extent because it does not root easily from cuttings and seed progeny are highly variable with respect to flower color. Blazich and Acedo (1988) reported successful micropropagation of flame azalea, which should contribute to its future availability for use in the landscape.

Propagation of flame azalea by tissue culture may provide large numbers of identical plants from a single shoot tip, but there are problems during microshoot acclimatization to greenhouse conditions. Plants grown in tissue culture often have morphological and physiological abnormalities

that are induced by the cultural conditions of high humidity and reduced light (Ziv, et al., 1987; Sutter, 1988). The most serious of these appears to be stomata that do not seem to have a closure mechanism and therefore are unable to close in response to a reduction in relative humidity. This results in plant desiccation and death (Brainerd and Fuchigami, 1981; Conner and Conner, 1984). Because of this lack of stomatal function in tissue-cultured microshoots, acclimatization is primarily a water status problem, due to potential desiccation, causing dehydration stress.

Potassium has long been considered the "water relations" nutrient most critical for proper maintenance of plant turgor and stomatal function (Mengel and Kirkby, 1987). While the actual mechanism of stomatal opening and closure is not yet completely understood, it has been established that the mechanism depends on K accumulation in the guard cells (Mengel and Kirkby, 1987). This ion accumulation reduces the osmotic potential of the guard cells and, as a result, water flows in and the turgid cells open (Kramer, 1983). Brag (1972) demonstrated that high K levels were directly correlated with reduced transpiration rate and stomatal opening in Triticum and Pisum. An addition of KCl to K-deficient wheat plants resulted in a decrease in transpiration rate of 50% in 2 hrs, and was attributed to changes in stomatal aperture. Eakes, et al. (1989) found that Salvia plants were able to better withstand moisture stress due to osmotic adjustment, when fertilized with high levels of K. The leaves accumulated K, which likely contributed to the decreased osmotic potential within the cells. This reduction then enabled the cells to retain water and maintain turgidity, despite dehydration stress. Stomatal sensitivity to drought was also improved.

The influence of K levels on microshoot development and stomatal function has not been examined. It seems plausible that microshoot stomata might become more responsive during acclimatization when exposed to higher levels of K. K uptake by the plant tissue might reduce microshoot water potential *in vitro* so that when the microshoots are transplanted to a moist peat/vermiculite medium, water will move into the microshoots in response to the water potential gradient. Additionally, the microshoots may be able to adjust osmotically to dehydration stress.

The objectives of this study on desiccation during acclimatization were: (1) to determine whether *in vitro* KCl treatments enable microshoots to resist desiccation; (2) to determine the effect of *in vitro* K treatments upon stomatal function during acclimatization; (3) to determine how *in vitro* KCl treatments affect microshoot water potential during acclimatization; and (4) to determine what effect *in vitro* KCl treatments have upon subsequent rooting and growth.

MATERIALS AND METHODS

Establishment and Growth of Cultures

On 16 and 23 October, 1988, 60 actively growing shoot tips were excised from 3-month-old seedlings of flame azalea that had been maintained in the greenhouse and fertilized weekly with a solution containing 50 ppm N, 10 ppm P, 30 ppm K, and a modified Hoagland's micronutrient solution (Hoagland and Arnon, 1950) containing 5 ppm Fe-EDTA. The shoot tips were stripped of leaves and washed sequentially with warm running water for 20 min., a 0.2% solution of Ivory soap for 10 min. on a shaker, a second running tap water rinse for 15 min., a solution of 10% commercial bleach with .05% 'Tween 20' for 15 min. on a shaker, and 3 rinses in sterile distilled water. The shoot tips were blotted dry with sterile paper towels and trimmed at both ends, leaving explants 2 cm long. The explants were placed in groups of 4 in 500 ml Pyrex storage jars on 100 ml Woody Plant Medium (Lloyd and McCown, 1980), modified with 15 ppm 2ip [6(γ,γ-dimethylallylamino purine)], 80 ppm adenine sulfate, and 200 ppm NaH_2PO_4 (Blazich and Acedo, 1988), hereafter referred to as WPM+. In addition, sucrose was reduced from 2 to 1.6% to lessen microshoot dependency on an exogenous carbon source, and agar was increased from 0.6 to 0.7% to provide drier WPM+ (R.E. Veilleux, personal communication). The 15 culture vessels were sealed with Parafilm (American Can Company, Greenwich, CT) and placed in a Fisher low temperature incubator modified with 2 each of 40-watt cool and warm fluorescent bulbs which provided a light intensity of 60 uMol/m^2/sec. Photoperiod was set at 16 hr light/8 hr darkness; temperature was 23C.

Shoot elongation of axillary buds began 20 days after culture. Axillary tissue was highly proliferative, with clumps 2 cm in diameter forming within 60 days. Each clump was composed of 10-30 microshoots that were suitable for transplanting to cellpaks containing a peat/vermiculite medium. The cultures were replenished by dividing a single clump into 4 smaller pieces and subculturing onto fresh WPM+. The microshoots that developed from the axillary tissue were used for the studies described subsequently. Because of a concern about genetic variability in response to stress, a single clump was divided on 2 January, 1989, and subcultured repeatedly. There was enough tissue for use in the final study of KCl influence upon microshoot stomatal function and water potential.

Establishment of Duration and Range of In Vitro KCl Treatment

In February 1989, microshoot clumps were subcultured into individual 20x150 mm glass tubes containing WPM+ amended with 0, 50, 100, 200, or 300 mM KCl, with 9 replicates for each KCl level. The tubes were incubated under the same environmental conditions as the stock cultures, for 4, 8, or 12 days. For day 0 clumps, microshoots were excised directly from WPM+ and placed in cellpaks containing sterile medium composed of finely milled peat and fine vermiculite, mixed 1:1 by volume. On each designated day, 3 clumps were removed from each *in vitro* treatment and 3 excised microshoots from each clump were placed in 15-cell cellpaks. There was a total of 12 cellpaks, each divided into 3 rows of 5 *in vitro* KCl treatments, with the treatments randomly assigned to the rows. The cellpaks were sealed in 2-gallon clear plastic bags and arranged in a completely randomized design

on a greenhouse bench under 30% shade cloth, which permitted a photosynthetically active light level of 200 uMol/m^2/sec at full sun. After 28 days, the microshoots were evaluated for rooting. Values for microshoot water potentials were determined on day 0, using thermocouple psychrometers (Model 84-IV, JRD Merrill Specialty Equipment Co., Logan, UT). The microshoot tissue in the psychrometers took 6 hrs to equilibrate in a constant temperature water bath at 30C. Water potential values were determined with a Wescor microvoltmeter (Logan, UT).

Percent Tissue K and Water Potential of 0 - 60 mM KCl Treatments

When it was determined that microshoots did not grow well after being cultured at *in vitro* KCl levels above 50 mM, the treatment range was adjusted to 0, 30, and 60 mM. Microshoot clumps were placed on the culture medium so that the shoot portion did not come in contact with the medium. After 9 days of subculture at the new concentrations, water potential values were determined in the manner described previously. To prepare the microshoot tissue for tissue K analysis, the shoot portion of the microshoot clumps was carefully harvested and thoroughly rinsed in distilled water. The tissue was dried for 48 hrs in a convection oven at 60C, weighed on a precision balance, then analyzed for percent tissue K by atomic absorption spectrophotometry.

In Vitro KCl Treatment and Reduced Relative Humidity

On 9 August, 1989, microshoots that had been subcultured on WPM+ con-

taining 0, 30, or 60 mM KCl for 9 days were weighed on a precision balance, then transferred to cellpaks containing 1:1 peat/vermiculite and sealed in 1 gallon clear plastic bags. The experimental design was a completely randomized design with twenty one 9-cell cellpaks (experimental units) each containing 3 microshoots (subsamples) from each of the 3 KCl treatments, randomly assigned to the rows. After 3 days on a greenhouse bench under 60% shade cloth which permitted a photosynthetically active light level of 250 uMol/m^2/sec at full sun, the microshoots were removed from the plastic bags and exposed for different periods to a floor fan that reduced the relative humidity surrounding the microshoots from 100% in the plastic bags to 79%. There were 6 exposure times, each replicated 3 times. Exposure times to the fan were 0.5, 1, 1.5, 2, 3, and 4 hrs, in addition to 3 controls that remained bagged. Following the fan exposure, the cellpaks were rebagged and placed back under the shade cloth for an additional 25 days. The microshoots then were removed from the cellpaks and those with roots were rinsed free of medium. The microshoots were immediately weighed on a precision balance. A tally was made of those microshoots that had rooted and percent fresh weight gain was calculated, by subtracting microshoot initial fresh weight from final fresh weight, then dividing by initial fresh weight. The percentage of microshoots that rooted and percentage of microshoot fresh weight gain was statistically analyzed by regression analysis, using the PROC GLM procedure of SAS (SAS Institure, Box 8000, Cary, NC).

In Vitro KCl Treatment and Desiccation in Isopiestic Tubes

A method of generating water release curves was needed to assess the effect of *in vitro* KCl upon microshoot capacity to resist desiccation, by osmotic adjustment. The stems of the microshoots were too small to fit into a Scholander pressure chamber (Scholander et al., 1965). Several attempts were made to construct water release curves for microshoots using thermocouple psychrometers. Microshoots were placed in the psychrometer chambers and after a 3 hr equilibration at 30C, water potentials were recorded. The psychrometers were then frozen in liquid nitrogen to destroy turgor potential. After the psychrometers were equilibrated to 30C again, a second reading was made, which should have been the osmotic potential. However, the values for osmotic potential were always less negative than those for microshoot water potential, indicating that turgor potential had a negative value. Therefore, this method could not be used to determine the components of microshoot water potential.

An isopiestic equilibration method developed by Livingston and de Jong (1988) to generate water release curves for leaf discs was used in an attempt to establish similar curves for microshoots. Livingston and deJong's method required that leaf discs be sealed in test tubes containing different unsaturated salt solutions ranging from 0 to -4.67 MPa. After the weight of the discs stabilized, usually in 12 hrs, they were considered to be in vapor equilibrium with, and at the same water potential as, the salt solutions. Water release curves were then obtained by plotting the paired values of disc relative water content and the inverse of the solu-

tion water potential.

Initially, a single salt solution was used to determine if this method could be applied to microshoots. Three microshoot clumps from each of the 3 in vitro KCl treatments individually were placed in isopiestic tubes, in foil baskets suspended above filter paper saturated with 20 ml of a 1 m NaCl solution. The tubes were sealed tightly with Parafilm and placed in a refrigerator at 5C to minimize cellular respiration. Periodically the clumps were removed from the tubes and quickly weighed. However, the weight loss of the clumps never stabilized, even after 10 days. Thus, a water release curve could not be plotted for the microshoot clumps.

The mean values for weight loss during exposure to the 1 m NaCl solution were plotted for the 3 replicates from each KCl treatment, and used to evaluate the effect of in vitro KCl upon microshoot weight loss. The regression lines for the percent weight loss of the 3 KCl treatments over time were compared by testing for homogeneity of the slopes, using the PROC GLM procedure of SAS.

In Vitro KCl Treatments, Stomatal Function, and Water Potential

On 29 January, 1990, WPM+ plus 0 mM, 30 mM, or 60 mM KCl was prepared and distributed to 20x150 mm glass flat-bottomed tubes at a volume of 20 ml per tube. The microshoots were incubated for 9 days under the same environmental conditions as previous cultures.

Following the *in vitro* incubation, individual microshoots were excised from the clumps and inserted into 9-cell cellpaks, 1 microshoot per cell. The cellpaks were divided into rows of 3 *in vitro* KCl treatments each, with the treatments randomly assigned to the rows. Three microshoots per cell were used for the water potential experiment, as earlier work showed that the most consistent readings were obtained with 3 microshoots per psychrometer chamber. The cellpaks were sealed in 1-gallon clear plastic bags and placed under 30% shade cloth in the greenhouse.

The stomatal function and water potential experiments were arranged in a completely randomized design using 2 cellpaks, one stressed, one not stressed, and evaluated on each of 3 days (9, 22, and 38 days for stomatal function, and 9, 21, and 39 days for water potential). Microshoots were also evaluated on day 0, when they were removed directly from culture, and either stressed or not stressed before measurements were made. The data from these two experiments were statistically analysed by regression analysis with the PROC GLM procedure of SAS.

Two cellpaks each for days 9, 22, and 38 were taken to the laboratory for evaluation of stomatal function. One cellpak was removed from the plastic bag and placed under a recirculating fan in an environmental growth chamber for 15 min. at a relative humidity of 50%, as measured with a wet bulb psychrometer. For day 0 evaluation, microshoot clumps were removed from the culture tubes and placed on petri plates prior to imposing stress. Otherwise they were handled in the same way as the microshoots in the cellpaks. Percent stomatal closure was measured by excising microshoot leaves,

placing them on a glass slide to which a drop of distilled water had been added, and counting the number of stomata that were completely closed in relation to the total number of stomata in the microscope field (400X). Nine counts for each of 3 microshoots were used (3 microscopic fields from each of 3 leaves per microshoot) for each KCl treatment at each of the 2 stress levels.

Water potential was recorded on days 0, 9, 21, and 39. As with the stomatal function experiment, 2 cellpaks each for Days 9, 21, and 39 were taken from the greenhouse, whereas day 0 microshoots were removed directly from culture. Three microshoots from each cellpak cell were placed in a single thermocouple psychrometer. The psychrometers then were equilibrated for 6 hours at 30C in a water bath and read with a Wescor microvoltmeter.

Acclimatization

On day 23, the plastic bags were removed from all cellpaks for 1/2 hour to expose the microshoots to the reduced humidity of the greenhouse. Sequentially, each day this exposure was increased by 1 hour, until day 30, when the microshoots were considered fully acclimatized. From day 20 on, those microshoots in the rooting experiment that had roots were fertilized with 50 ppm N to maximize growth for the percent weight gain data.

RESULTS

Establishment of Duration and Range of In Vitro KCl Treatment

Microshoots were subcultured onto WPM+ containing a wide range of KCl concentrations, then evaluated for water potential and root development. This was done to establish KCl treatment levels and duration of *in vitro* exposure.

In general, microshoot water potentials decreased as *in vitro* KCl levels increased from 0 to 300 mM KCl. By day 4 of *in vitro* exposure, average water potential values decreased from -1.34 MPa for controls to -1.82 MPa for 50 mM, -2.31 MPa for 100 mM, and -3.40 MPa for 200 and 300 mM KCl treatments. Similar water potential values were recorded for KCl treatments after 8 and 12 days of *in vitro* exposure.

The percentage of microshoots that rooted by day 28 *ex vitro* generally decreased as the level of *in vitro* KCl increased (Figure 2.1). Microshoots exposed to 200 and 300 mM KCl for 12 days *in vitro* died within a few days after transfer to peat/vermiculite medium. Therefore, for all subsequent experiments, *in vitro* KCl concentrations of 0, 30 and 60 mM were used, and *in vitro* exposure was limited to 9 days.

Percent Tissue K and Water Potential of 0 - 60 mM KCl Treatments

After 9 days of subculture on 0, 30, or 60 mM KCl treatment, micro-

shoots were analyzed for tissue K and microshoot water potentials were determined. As the level of K increased in the *in vitro* treatment from 0 to 60 mM, percent tissue K increased from an average value of 3.4% for controls to 6.3% for 30 mM KCl and 8.7% for 60 mM KCl. Water potentials decreased with increased KCl level, dropping from an average value of -0.26 MPa for controls to -0.69 MPa for 30 mM KCl and -1.05 MPa for 60 mM KCl (Figure 2.2).

In Vitro KCl Treatment and Reduced Relative Humidity

Microshoots were exposed to a fan for periods ranging from 0.5 to 4 hrs, 3 days after transfer from *in vitro* KCl treatment to peat/vermiculite medium. Neither the *in vitro* KCl treatment nor the length of fan exposure had a significant effect upon the number of microshoots that rooted, or the percent fresh weight gain (Table 2.1).

In Vitro KCl Treatment and Desiccation in Isopiestic Tubes

Microshoot clumps subcultured for 9 days on *in vitro* KCl treatments were placed in isopiestic tubes containing a 1 m NaCl solution with an osmotic potential of -4 MPa. Livingston and deJong (1988) had obtained weight equilibration of leaf discs in similar tubes after 12 hrs, and were able to construct water release curves from which they calculated osmotic and turgor potentials for the leaf tissue. We were unsuccessful in duplicating their results; after 10 days in the isopiestic tubes, the microshoot clumps were still losing weight. Equilibration of clumps in tubes

containing osmotic solutions of -0.92 and -2.9 MPa also failed, again since weight stabilization never occurred. It is likely that the microshoots continued to respire during the isopiestic incubation, losing tissue mass, which may have masked the vapor equilibration between the solution and the tissue. The percent weight loss for microshoots from each *in vitro* KCl treatment was plotted over the 10-day period (Figure 2.3). The slopes of the regression lines for microshoot weight loss were not significantly different from each other, indicating that *in vitro* KCl level did not influence microshoot weight loss.

In Vitro KCl Treatments, Stomatal Function, and Water Potential

Microshoots subcultured on *in vitro* KCl treatments were evaluated for stomatal function and water potential on different days following transfer to peat/vermiculite medium. Half of the microshoots were exposed to a reduced relative humidity for 15 min. before evaluation. Percent stomatal closure increased as days *ex vitro* increased, and in response to dehydration stress, with 1 exception (Table 2.2). On day 22, the average percent stomatal closure was lower than on day 9 for both 0 and 30 mM KCl treatments. These are likely to be abnormal values, as there is no biological basis for stomatal function to decrease on this particular date.

Regression analysis of the effect of *in vitro* KCl treatments, days *ex vitro*, and 15 min. dehydration stress upon percent stomatal closure indicates that there are significant interactive effects between KCl treatment, days *ex vitro*, and stress (Tables 2.2, 2.3).

The true effect of *in vitro* KCl on percent stomatal closure will more likely be revealed when the microshoots are stressed. While the microshoots remained in a nonstressful situation, stomatal behavior could be highly variable and likely dependent upon the physical and physiological environment immediately surrounding the guard cells. When the entire microshoot was subjected to a desiccating stress, the whole plant response would be a unified reaction to the stress, and any treatment differences would manifest themselves under these conditions. The analysis of the percent stomatal closure values of only stressed microshoots showed that days *ex vitro* had a highly significant effect, but *in vitro* KCl treatment did not. Interactive effects were not significant.

At designated times throughout the 39-day acclimatization period, one half of the microshoots from the 3 KCl treatments received a 15 min. exposure to reduced relative humidity, then all were placed in thermocouple psychrometers to determine water potentials. Both *in vitro* KCl treatments and exposure to dehydration stress significantly reduced microshoot water potentials. Water potential values generally increased as days *ex vitro* increased (Figure 2.4). Considering that microshoot water potentials were reduced by increased levels of *in vitro* KCl, the interactive effect of days *ex vitro* and KCl treatment is likely due to an abnormally high value for unstressed 30 mM KCl treatments at day 0 (Table 2.2).

DISCUSSION

Microshoots cultured on high levels of *in vitro* KCl showed increased tissue K content, with a concomitant reduction in microshoot water potential. It is likely that K accumulated by the microshoots decreased osmotic potential within the tissues, based on the work of Eakes et al. (1988). Bar-Tsur and Rudich (1987) demonstrated that K uptake and reduced osmotic potential in cotton plants resulted in osmotic adjustment that enabled the plants to remain turgid during water stress. Whether the reduction in microshoot water potential could improve microshoot resistance to desiccation specifically via osmotic adjustment is unknown, as water release curves could not be constructed for the microshoots from the isopiestic study. Other methods for determining osmotic potential (Scholander pressure chamber, thermocouple psychrometer) either were not usable with microshoots, or failed to give reasonable values.

Mengel and Arnecke (1982) have shown that high K availability in the medium solution increases solute accumulation by the plant and decreases root water potential, thus providing a water potential gradient and stimulating water uptake. Although microshoots had not rooted by day 3 *ex vitro*, when the fan stress was imposed, the more negative water potential of the microshoot stem in relation to the surrounding medium still would establish a water potential gradient. Enhanced water uptake during a desiccating stress, such as a prolonged fan exposure, would likely improve the chances for microshoot survival. However, microshoots subcultured on 30 and 60 mM KCl did not have a better survival rate when subjected to dif-

ferent fan exposures, as measured by the percentage of microshoots that had rooted by day 28 (Table 2.1).

It is likely that the increase of stomatal function over 38 days ex vitro (Table 2.2) enabled the microshoots to control transpiration, resulting in less tissue desiccation. As tissues were able to remain hydrated, especially after rooting improved hydraulic conductance into the stems, water potentials became less negative.

Microshoot water potentials were lower when cultured at the higher in vitro KCl levels, but this effect was moderated by the length of time the microshoots were out of culture (Figure 2.4). As days ex vitro increased, microshoot water potentials became less negative, rising to an average of -0.6 MPA for the 3 KCl treatments by day 39. This increase in water potential over time was paralleled by an increase in stomatal function, as well as the development of roots around day 16 ex vitro. Continued shoot development likely initiated auxin synthesis, which enabled the vascular elements to be formed at the root-shoot juncion (Galston, et al., 1980). The continuity of the xylem tissue would facilitate water transport from roots to shoots.

Rein et. al. (1990) observed a similar increase in water potential values over time in cuttings from 3 species of woody ornamentals. Stem cuttings of juniper cv. 'Blue Rug' had water potential values around -6 MPa 14 days after sticking in a peat/perlite propagation medium (125% moisture level). Fourteen days later, the water potentials of the cuttings had

risen to -4.7 MPa. This increase was attributed in part to the rooting of the cuttings.

The lack of influence of KCl on percent stomatal closure suggests that mechanisms other than those related to K control of stomata are involved with microshoot stomatal function. If the stomata had been already functional, then a positive influence of K might have been observed. However, since in vitro KCl treatments did not influence percent stomatal closure even at day 38 when stomata were functional, it is assumed that sufficient K is supplied by WPM+ alone. The sufficiency of K levels in WPM+ is further demonstrated by the lack of a positive influence of K on microshoot rooting and percent fresh weight gain.

The development of functional stomata was dependent only on the length of time the microshoots had been out of culture. During acclimatization, microshoots most likely underwent physiological and morphological changes associated with rooting and exposure to an environment that promoted autotrophic development, which in some way increased stomatal function. Cytokinins produced in the roots have been shown to directly affect the development of leaf mesophyll tissue (Wareing and Phillips, 1970); there may be a direct connection between cytokinin activity and stomatal function as well. Marin et. al. (1988) studied the influence of acclimatization on stomatal structure and function of micropropagated cherry, especially stomatal response to reduced relative humidity over time. They found several histochemical differences between in vitro and greenhouse-grown plants that may relate to ontological development. For example, the

continuous presence of starch in guard cell chloroplasts from unacclimatized *in vitro* plants contrasted with the disappearance of starch in guard cell chloroplasts of greenhouse plants when the stomata were closed. When the tissue-cultured cherry plants were exposed gradually to reduced relative humidity, the starch disappeared from chloroplasts of those guard cells that became functional. Thus, there may be factors such as starch degradation in chloroplast guard cells that determine the development of stomatal function.

In summary, *in vitro* KCl treatments induced elevated tissue K levels in microshoots and reduced microshoot water potentials, but it could not be shown that these effects conferred any benefit with respect to avoidance of desiccation. Microshoot maturation, as a function of days out of tissue culture, had the greatest effect on the development of stomatal function, which, coupled with the onset of rooting, improved microshoot water status.

These results indicate that successful acclimatization depends on the length of time that microshoots are out of culture. Future research should continue to focus on developing an understanding of stomatal function and what causes stomata to develop abnormally (nonoperational). Until this understanding is complete, regimens that enable microshoots to maintain a stable water status during acclimatization will be necessary. Programs that could hasten rooting or induce earlier stomatal function should continue to be tested.

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Table 2.1. The effect of *in vitro* KCl treatment and fan exposure upon percent microshoots rooted and microshoot percent fresh weight gain.

KCl (mM)	Fan exposure (hrs) 0	0.5	1	1.5	2	3	4	$\bar{X}$
				Percent Rooted				
0	66[z]	33	66	66	33	22	22	44
30	44	33	44	66	66	22	33	43
60	55	44	44	22	22	33	11	33
$\bar{X}$	55	37	51	51	40	26	22	
				Percent Fresh Weight Gain				
0	310[y]	359	679	141	284	50	270	299
30	569	236	272	170	117	86	238	241
60	193	295	147	271	154	178	512	250
$\bar{X}$	357	297	366	194	185	105	340	

Regression Analysis

	% MS rooted	% Fresh weight gain
in vitro KCl Treatment	NS	NS
Fan exposure	NS	NS
Trt x Exposure	NS	NS

[z] Mean rooting percentage for 9 microshoots, evaluated after 28 days.

[y] Mean value for percent fresh weight gain of microshoots that rooted, calculated as final fresh weight - initial fresh weight / initial fresh weight x 100.

Table 2.2. Effect of *in vitro* KCl treatment, days *ex vitro*, and dehydration stress on percent stomatal closure and water potential of microshoots of flame azalea.

KCl (mM)	Days *ex vitro*		Stomatal closure (%) no stress	stress	$\bar{X}$		Water potential (MPa) no stress	stress	$\bar{X}$
0	0		8.1[z]	18.1	13.1[y]		-.52[x]	-1.33	-.93[y]
	9		19.1	44.2	31.7		-.70	-1.23	-.97
	22		8.9	76.4	42.7		-.66	-1.40	-1.03
	38		80.7	82.7	81.7		-.39	-.55	-.47
		$\bar{X}$	29.2[w]	55.4	42.3	$\bar{X}$	-.57	-1.13	-.85
30	0		15.2	16.6	15.9		-.39	-1.12	-.76
	9		50.2	68.8	59.5		-1.42	-1.99	-1.70
	22		2.4	86.2	44.3		-1.00	-1.95	-1.48
	38		77.2	73.8	75.5		-.31	-.97	-.64
		$\bar{X}$	36.3	61.4	48.9	$\bar{X}$	-.78	-1.51	-1.25
60	0		5.3	3.9	4.6		-.84	-2.64	-1.74
	9		47.1	35.8	41.4		-1.74	-2.12	-1.93
	22		52.7	70.9	61.8		-1.29	-1.33	-1.31
	38		92.7	83.8	88.3		-.45	-1.11	-.78
		$\bar{X}$	49.5	48.5	49.0	$\bar{X}$	-1.08	-1.80	-1.44

Regression Analysis

	Stomatal closure stressed + nonstressed	Stomatal closure stressed only	Water potential
days *ex vitro*	**** [v]	****	****
in vitro KCl trt	*	NS	***
dehydration stress	****	-	****
days x trt	**	NS	*
days x stress	****	-	NS
stress x trt	**	-	NS
days x stress x trt	NS	-	NS

[z] Mean of 3 microshoots, 3 leaves per microshoot, 3 microscopic fields per leaf.
[y] Mean of days x trt values.
[x] Mean of 3 psychrometer readings.
[w] Mean of stress x trt values.
[v] NS, *, **, ***, **** Nonsignificant and significant at the .05, .01, .001, and .0001 levels, respectively.

Table 2.3. The effect of days ex vitro and exposure to dehydration stress upon mean percent stomatal closure in microshoots of flame azalea.

Treatment	Days ex vitro 0	9	22	38	$\bar{X}$
No stress[z]	9.5[x]	38.8	21.3	83.5	38.3
Stress[y]	12.9	49.6	77.8	80.1	55.1
$\bar{X}$	11.2	44.2	49.6	81.8	

[z] Leaves were excised from microshoots without exposure to dehydration stress and percent stomatal closure determined.

[y] Leaves were excised from microshoots that had been exposed to 15 min. dehydration stress, and percent stomatal closure determined.

[x] Mean of values for percent stomatal closure of 3 KCl treatments for a given day ex vitro, and separated by stress treatment.

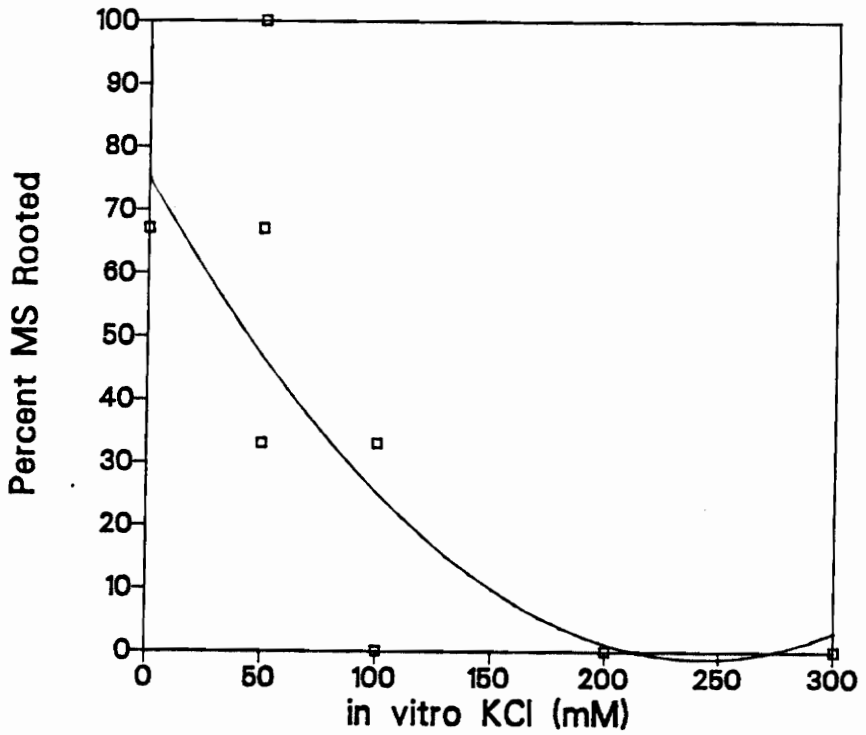

Figure 2.1. The effect of *in vitro* KCl exposure for 12 days upon percent microshoots rooted. Nine microshoots were subcultured at each KCl concentration (3 blocks with 3 replications in each block). Regression equation: $Y = 0.75 - 0.0063X + 0.000013X^2$. $R^2 = .72$.

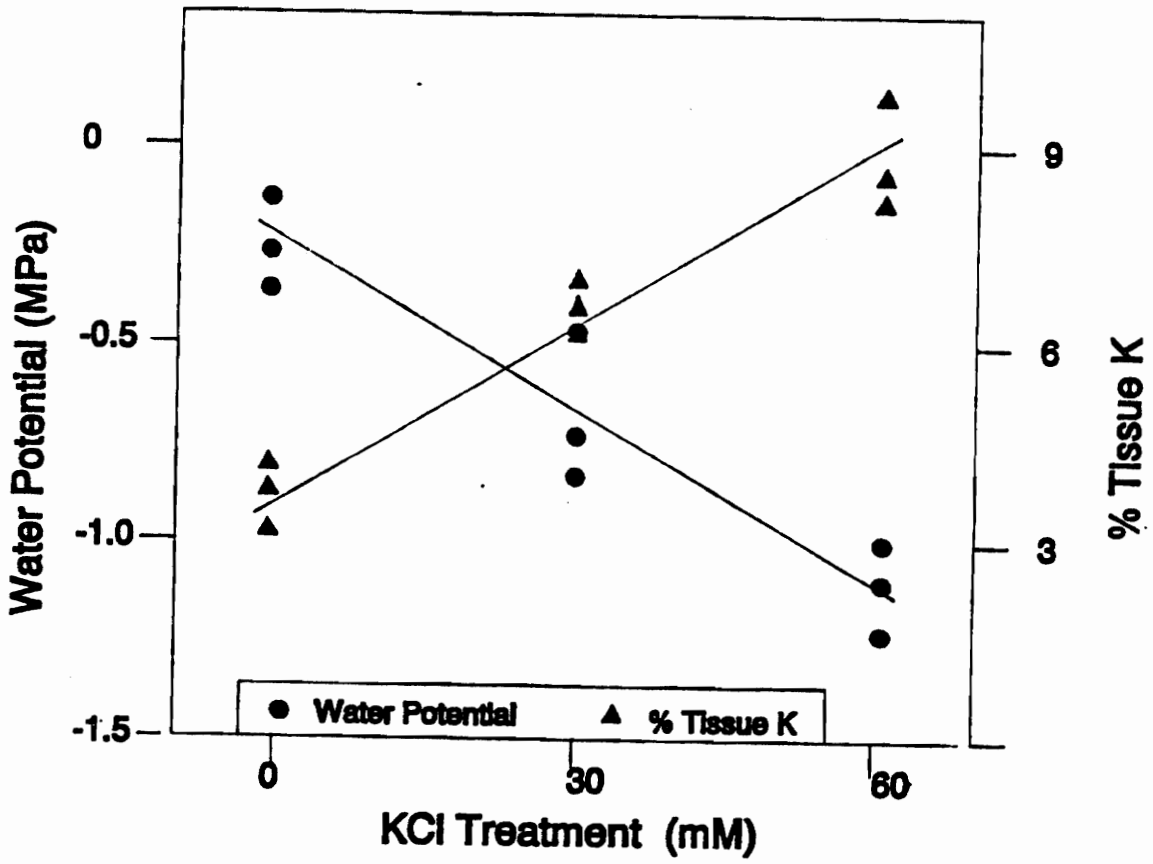

Figure 2.2. The effect of 0, 30, and 60 mM KCl *in vitro* treatments upon % tissue K and microshoot water potential. Each data point for the water potential line represents 3 microshoots taken from a single clump and placed in a single psychrometer. Each data point for the % tissue K line represents at least 25 mg combined dried weight of no less than 10 clump replicates. Regression equations: (water potential) $Y = -0.268 - 0.0013X$. $R^2 = .86$. (% tissue K) $Y = 3.45 + 0.089X$. $R^2 = .96$.

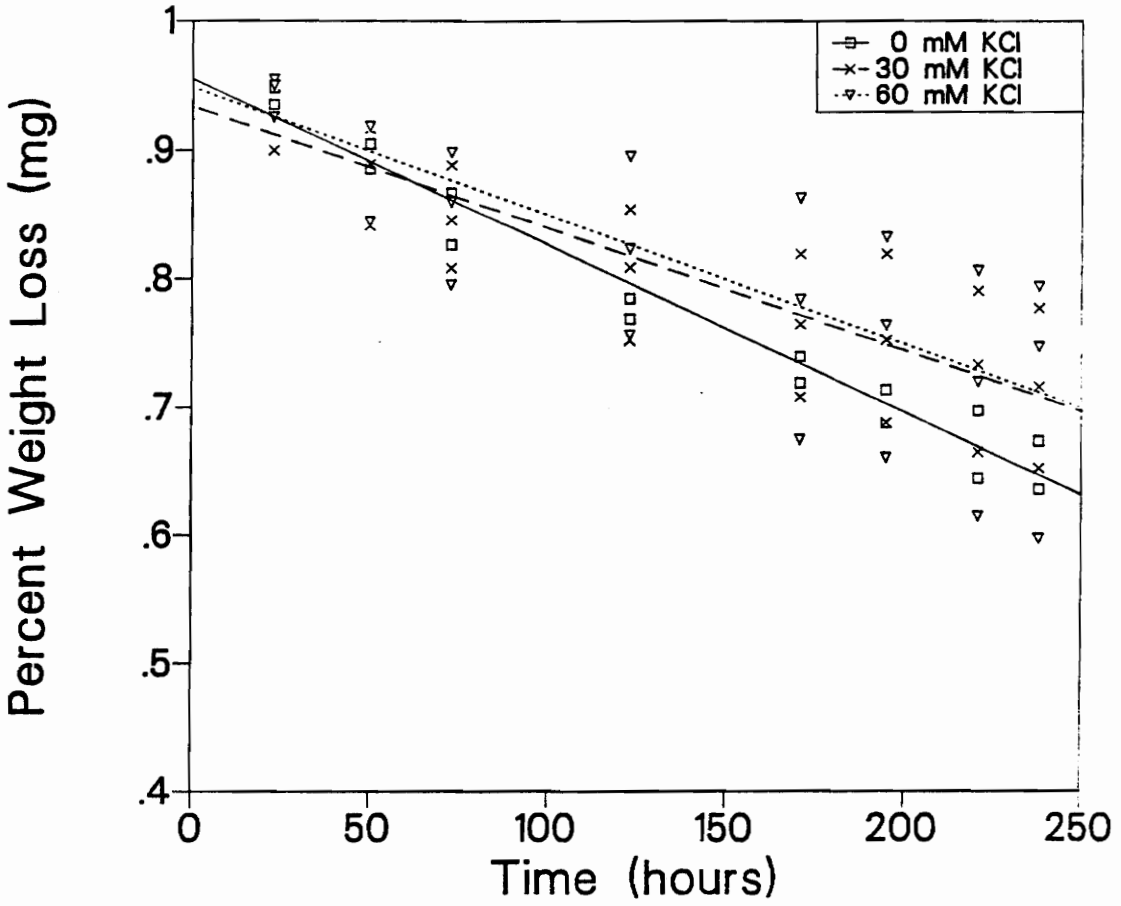

Figure 2.3. Percent weight loss in isopiestic tubes over 10 days for microshoot clumps from 3 in vitro KCl treatments. Regression equations: (0 mM KCl) Y = 0.956 - 0.0013X. R^2 = .97. (30 mM KCl) Y = 0.934 - 0.00095X. R^2 = .73. (60 mM KCl) Y = 0.949 - 0.001X. R^2 = .61.

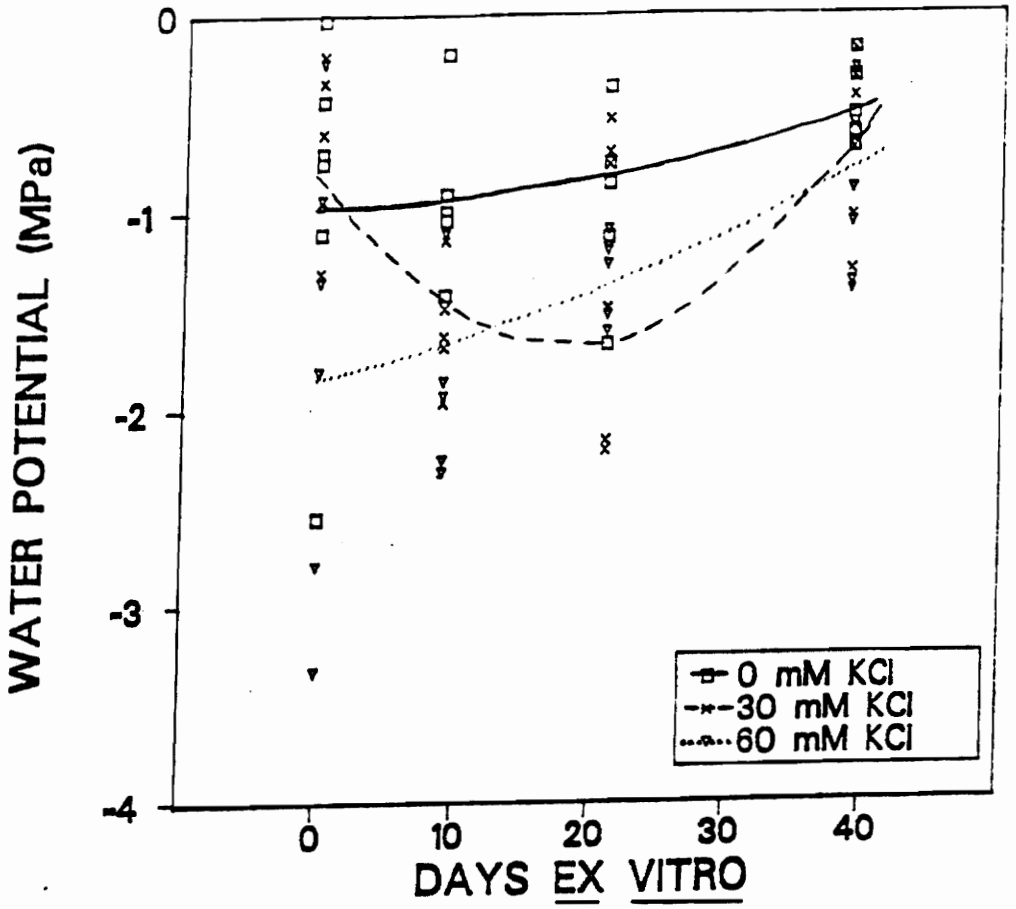

Figure 2.4. Water potentials for 3 *in vitro* KCl treatments at 4 different times during acclimatization. At Day 0, microshoots were taken directly from tissue culture and water potential measured; at Days 9, 21, and 39 microshoots were excised from cellpaks and water potential measured. Regression equations: (0 mM KCl) $Y = -0.972 + 0.00032X^2$, $R^2=.12$; (30 mM KCl) $Y = -0.815 - 0.093X + 0.0025X^2$, $R^2=.45$; (60 mM KCl) $Y = -1.767 + 0.0007X^2$, $R^2=.30$.

VITA

Lucy Gray Taylor was born on January 27, 1950, in Roanoke, Virginia. She received her secondary education in New York but returned to the South to complete a B.A. in Biology at Hollins College in 1972. She has been employed as an elementary and junior high school teacher, personnel manager for various manufacturers in the southeastern U.S., and medical researcher in the areas of adult onset diabetes and bacterial enzyme kinetics.

In 1987, the author fulfilled a lifelong dream of continuing her education in the life sciences by enrolling in the Master of Science program in Horticulture. She is presently employed as a laboratory specialist in postharvest physiology with the Department.

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