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The effect of irradiance levels during in vitro propagation on leaf anatomy and plantlet survivability

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The effect of irradiance levels during in vitro propagation on leaf anatomy and plantlet survivability

by

Sheryl Kay Dunston

A Thesis Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE

Major: Horticulture

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1983
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENERAL INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>Explanation of thesis format</td>
<td>3</td>
</tr>
<tr>
<td><strong>REFERENCES CITED</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>SECTION I. IN VITRO PROPAGATION OF MARANTA LEUCONEURA 'KERCHOVIANA'</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>ASEPTIC CULTURE</strong></td>
<td>10</td>
</tr>
<tr>
<td><strong>EXPERIMENTS</strong></td>
<td>13</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>13</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>13</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>16</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>24</td>
</tr>
<tr>
<td><strong>REFERENCES CITED</strong></td>
<td>27</td>
</tr>
<tr>
<td><strong>SECTION II. A COMPARISON OF THE LEAF ANATOMY OF MICROPROPAGATED SHOOTS, ACCLIMATIZED PLANTLETS, AND GREENHOUSE-GROWN PLANTS</strong></td>
<td>28</td>
</tr>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>29</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>30</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>32</td>
</tr>
<tr>
<td>Aseptic culture</td>
<td>32</td>
</tr>
<tr>
<td>Acclimatization procedures</td>
<td>32</td>
</tr>
<tr>
<td>Greenhouse culture</td>
<td>33</td>
</tr>
<tr>
<td>Leaf anatomy</td>
<td>33</td>
</tr>
</tbody>
</table>
SECTION III. THE EFFECTS OF VARIOUS PHOTOSYNTHETIC PHOTON FLUX DENSITIES ON THE LEAF ANATOMY AND CHLOROPHYLL CONTENT OF TISSUE-CULTURED PLANTS

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

Aseptic culture

Irradiance treatments

Leaf anatomy

Leaf chlorophyll content

RESULTS

DISCUSSION

REFERENCES CITED

SECTION IV. THE EFFECTS OF VARIOUS PHOTOSYNTHETIC PHOTON FLUX DENSITIES IN VITRO AND SUBSEQUENT ACCLIMATIZATION TECHNIQUES ON THE SURVIVABILITY OF TISSUE-CULTURED PLANTS

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

Aseptic culture

Irradiance treatments

Acclimatization treatments

Experimental design

Statistical analysis
GENERAL INTRODUCTION

Micropropagation has become increasingly important in the multiplication of a variety of plants. Through tissue culture, it has become feasible to rapidly propagate plants which are slow or difficult to propagate by conventional means (15). *In vitro* culture also provides a means of expediently producing pathogen-free stock material (15).

Successful micropropagation procedures have been developed for many ornamental plants; however, success *in vitro* does not ensure continued vigorous growth in the greenhouse (15). Many plantlets transferred from tissue culture vessels into greenhouse environments die or exhibit a decrease in vigor (18). Poor survival rates of aseptically-cultured carnation (5, 18), blackberry (3), and rose (13, 16) plantlets have been reported. Tissue-cultured plantlets exist in an environment of nearly 100% relative humidity and relatively low irradiance levels. When plantlets are removed from the *in vitro* environment and placed into soil in the greenhouse, they are subjected to higher irradiance levels and a lower relative humidity than in the culture vessel.

Several methods of acclimatizing tissue-cultured plantlets have been developed. Humidity tents and/or intermittent mist systems offer intermediate humidity and irradiance levels (4, 14, 17). Such procedures often convert micropropagated shoots and plantlets into autotrophic plants, able to withstand the greenhouse environment and begin rapid growth (12). However, plant loses of some species remain unacceptably high.

Numerous research projects have investigated possible causes for the
high mortality rate while others have sought possible solutions. Desiccation and wilting are thought to be major factors contributing to these low survival rates (5, 9, 18). High relative humidities within culture vessels are thought to inhibit epicuticular wax formation in cauliflower (9), carnation (18), and 'Pixy' plum (7) which leads to excessive transpiration when the plantlets are transferred to lower relative humidities in the greenhouse. Tissue desiccation also may be due to malfunctioning of the stomatal closure mechanism (1, 7, 18). It has been reported that slow stomatal response in aseptically-cultured apple leaves leads to excessive water loss (1). Low photosynthetic rates at the time of transplanting are thought to contribute to the vulnerability of aseptically-cultured plantlets. Cauliflower plantlets derived by tissue culture were found to have lower Hill reaction activity, carbon dioxide fixation rates, and chlorophyll levels than cauliflower seedlings (11).

Few studies have been conducted to determine the effect of in vitro culture and acclimatization procedures on internal leaf anatomy. The leaf anatomy of micropropagated cauliflower (8, 9), 'Pixy' plum (2), and sweetgum (19) plantlets was found to be significantly different from those of acclimatized or greenhouse-grown plants. Leaves developed within culture vessels lacked well defined palisade layers and had an increase in the amount of mesophyll air space (2, 10, 19).

Leaf anatomy is influenced by soil moisture, humidity and light (6). Since most work has concentrated on the effects of humidity on leaf structure, these studies investigated the role of light. The overall objectives of this research are to investigate the effects of various
photosynthetic photon flux densities (PPFD) levels in vitro and of acclimatization techniques on internal leaf anatomy, growth rates, and survivability of tissue-cultured plantlets.

Explanation of thesis format The individual sections of this thesis are written for submission to scientific journals. The general introduction and overall summary have been included to unify the individual research efforts, and, therefore, help the reader assimilate the results.

Section I, "Micropropagation of Maranta leuconeura 'Kerchoviana'" presents a discussion of the selection of growth regulators and their appropriate concentrations required to stimulate root and shoot formation. This article has been written for publication in HortScience.

Section II is an investigation of the differences in the anatomy of leaves excised from micropropagated shoots, acclimatized plantlets, and greenhouse-grown cuttings. 'A comparison of the leaf anatomy of micropropagated shoots, acclimatized plantlets, and greenhouse-grown plants' has been prepared for publication in HortScience.

'The effects of various photosynthetic photon flux densities in vitro on the leaf anatomy of tissue-cultured plants', Section III, has been formatted for publication in the Journal of the American Society for Horticultural Science. In this study, leaves excised from aseptically-cultured plantlets grown under different irradiance levels were compared.

Section IV is an examination of the effects of various irradiance levels in vitro and subsequent acclimatization techniques on relative growth rates and survivability of plants following transfer to the
greenhouse environment. The results of this study have been assimilated into an article entitled, 'The effects of various photosynthetic photon flux densities in vitro and subsequent acclimatization techniques on the survivability of tissue-cultured plants', written for publication in the Journal of the American Society for Horticultural Science.
REFERENCES CITED


SECTION I. IN VITRO PROPAGATION OF MARANTA LEUCONEURA 'KERCHOVIANA'
ABSTRACT

Lateral buds excised from unrooted cuttings of *Maranta leuconeura* 'Kerchoviana' were cultured on full-strength Linsmaier and Skoog (LS) medium supplemented with various combinations of benzyladenine (BA), kinetin, naphthaleneacetic acid (NAA), and indoleacetic acid (IAA). After a preliminary screening of 48 combinations, the combinations that produced the most vigorous, well-formed plants were studied further: 2.0 mg/liter kinetin, 0.2 mg/liter BA, 0.2 mg/liter BA and 0.1 mg/liter NAA, and 2.0 mg/liter kinetin and 1.0 mg/liter IAA. The best shoot development was obtained after subculturing shoots 12 weeks on LS medium supplemented with 0.2 mg/liter BA.
INTRODUCTION

*Maranta leuconeura* 'Kerchoviana' (prayer plant) is normally propagated asexually by stem cuttings because seeds rarely germinate (1). Cuttings are rooted in humidity tents for 4 - 6 weeks depending on the time of year. However, it is estimated that 15 - 20% of the cuttings produce inferior root systems and must be discarded.¹ Developing roots are unable to penetrate leaf sheaths uniformly and lateral root growth is inadequate.

The objective of this study was to develop *in vitro* techniques which produce attractive, compact plants with strong, fibrous root systems.

¹Tom Harcharik, Plant Manager, Yoder Brothers, Salinas, CA
ASEPTIC CULTURE

Rooted and unrooted cuttings (20 - 25 cm long) were used as an explant source. After leaf blades and roots were removed, stems were soaked in 20% commercial bleach plus 0.02% Triton® X-100 for 15 minutes with intermittent shaking. A second application of dilute bleach was applied for 5 minutes. Stems were transferred into sterilized jars and rinsed three times with sterile deionized water. Lateral buds (2 - 5 mm) were excised under aseptic conditions (Figure 1).

Lateral bud explants were placed in 25 X 150 mm culture tubes containing 15 ml of full-strength LS medium (3). The pH of the medium was adjusted to 5.8 prior to adding 8 g/liter Difco® Bacto-agar and autoclaving for 15 minutes at 121 C. Subsequent shoots were subcultured on 15 ml of LS medium in 25 X 150 mm culture tubes. Explants and subcultured shoots were incubated in controlled environment chambers at 26 ± 1 C under cool white fluorescent lights providing 78.5 ± 6 μEm⁻²s⁻¹ during a 16 hour photoperiod for shoot and root development.
Figure 1. An excised lateral bud, 2-5 mm in length.
EXPERIMENTS

Experiment 1  Combinations of exogenous auxins (NAA and IAA) and cytokinins (kinetin and BA) were compared for their effects on shoot and root development. Kinetin at 1, 2, 3, and 5 mg/liter and BA at 0.1, 0.2, 0.5, and 1.0 mg/liter were combined with NAA at 0, 0.1, and 1.0 mg/liter and IAA at 0, 1.0, and 2.0 mg/liter. Each of the 48 treatments was replicated five times on explants from both rooted and unrooted cuttings. Contamination of explants from rooted cuttings was 89% compared to 22% of those from unrooted cuttings. Only data on unrooted cutting explants were analyzed.

After 6 weeks in culture no statistically significant differences were found in the number of shoots or roots formed per treatment. However, large variations in plant morphology and overall vigor were observed. The four hormone treatments which produced the most vigorous and well-formed shoots were selected for further study.

Experiment 2  In the second experiment, 100 randomly selected lateral shoots (10 - 15 mm long) were subcultured in each of the four hormone treatments selected in Experiment 1. These treatments included the addition of 2.0 mg/liter kinetin, 0.2 mg/liter BA, 2.0 mg/liter kinetin and 1.0 mg/liter IAA, or 0.2 mg/liter BA and 0.1 mg/liter NAA to full-strength LS medium.

After 6 weeks, the number of lateral shoots and roots which formed per treatment did not vary significantly (Table 1). Lateral shoots subcultured on medium containing 0.2 mg/liter BA and 0.1 mg/liter NAA
Table 1. The effects of various hormone concentrations on shoot proliferation and root development.

<table>
<thead>
<tr>
<th>Hormone concn (mg/liter)</th>
<th>Mean no. $^z$ of shoots</th>
<th>Rating of $^y$ roots</th>
<th>Description of shoots and leaves</th>
<th>Description of roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 Kinetin</td>
<td>1.90</td>
<td>2.27</td>
<td>moderately sized shoots and leaves</td>
<td>thick, few lateral roots</td>
</tr>
<tr>
<td>0.2 BA</td>
<td>1.80</td>
<td>1.86</td>
<td>large shoots and leaves</td>
<td>thick, no lateral roots</td>
</tr>
<tr>
<td>2.0 Kinetin and 1.0 IAA</td>
<td>1.98</td>
<td>2.32</td>
<td>small shoots and leaves</td>
<td>some lateral roots</td>
</tr>
<tr>
<td>0.2 BA and 0.1 NAA</td>
<td>2.10</td>
<td>2.08</td>
<td>moderately sized shoots and leaves</td>
<td>many lateral roots</td>
</tr>
</tbody>
</table>

$^z$Mean of 100 lateral shoots 6 weeks after subculturing (S.E.M. = 0.14).

$^y$Roots rated on a numerical scale from 0 - 4: 0 = no roots, 1 = 1 - 5 roots, 2 = 6 - 10 roots, 3 = 11 - 15 roots, and 4 = more than 15 roots.
developed the most desirable shoots: the shoots were compact and had moderately-sized leaves.

Experiment 3  The optimum length of time required to produce the maximum number of shoots in vitro varies with different species (2). The purpose of this experiment was, therefore, to determine if a longer rapid multiplication phase (stage II) (4) was beneficial when propagating Maranta leuconeura 'Kerchoviana' by in vitro techniques. Twenty-four randomly selected lateral shoots (10 - 15 mm long) were subcultured on LS medium supplemented with 2.0 mg/liter kinetin, 0.2 mg/liter BA, 2.0 mg/liter kinetin and 1.0 mg/liter IAA, 0.2 mg/liter BA and 0.1 mg/liter NAA or medium without supplemental hormones.

After 12 weeks in culture, a significantly larger number of lateral shoots was produced on media supplemented with 0.2 mg/liter BA and 0.1 mg/liter NAA or 0.2 mg/liter BA than was produced in the other treatments (Table 2). Since these two treatments did not differ significantly, it is assumed that the addition of 0.1 mg/liter NAA is not necessary to obtain this level of proliferation. Overall plant shoot and root morphology and vigor was comparable. All cultures developed extensive root systems. When cultured 16 weeks, the shoots exhibited reduced vigor.

In addition, subcultured shoots with 2 - 3 leaves (Figure 2) were found to produce 5 - 6 lateral shoots in 12 weeks (Figures 3 and 4) when transferred to LS medium containing 0.2 mg/liter BA or 0.2 mg/liter BA and 0.1 mg/liter NAA. A reduction in vigor was observed when these shoots were subcultured for 16 weeks. When these lateral shoots were subcultured on LS medium devoid of supplemental hormones for 3 - 4 weeks, they
Table 2. The effects of various hormone concentrations on the number of shoots produced per subcultured lateral shoot.

<table>
<thead>
<tr>
<th>Hormone concn (mg/liter)</th>
<th>Mean no. of shoots$^z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 BA and 0.1 NAA</td>
<td>3.46</td>
</tr>
<tr>
<td>0.2 BA</td>
<td>3.92</td>
</tr>
<tr>
<td>2.0 Kinetin and 1.0 IAA</td>
<td>2.71</td>
</tr>
<tr>
<td>2.0 Kinetin</td>
<td>2.54</td>
</tr>
<tr>
<td>none</td>
<td>2.54</td>
</tr>
</tbody>
</table>

$^z$Mean number of shoots produced on 24 lateral shoots 12 weeks after subculturing (S.E.M. = 32).
Figure 2. Lateral shoot subcultured at the two-leaf stage.
Figure 3. Shoot development after twelve weeks in culture on LS medium supplemented with 0.2 mg/liter BA.

Figure 4. Individual shoots produced from one lateral bud explant in twelve weeks.
produced strong, fibrous root systems (Figure 5) and were easily established in the greenhouse environment.
Figure 5. Root development four weeks after subculturing lateral shoot at the two-leaf stage onto Linsmaier-Skoog medium without supplemental hormones.
DISCUSSION

Under the conditions of these experiments, the best substrate for in vitro production of lateral shoots of *Maranta leuconeura* 'Kerchoviana' was a full-strength LS medium supplemented with 0.2 mg/liter BA. Almost twice as many shoots were produced in 12 weeks as compared to 6 weeks indicating that the 12 week growth period would be superior for shoot production. Shoots subcultured at the 2 leaf stage on LS medium containing 0.2 mg/liter BA produced 5 - 6 lateral shoots in 12 weeks. Stage II shoots produced sufficient roots in 3 - 4 weeks on LS medium without supplemental hormones and the resultant stage III plantlets were transferred into the greenhouse environment without difficulty.

Prayer plants derived from tissue culture are compact plants (Figure 6) suitable for use in terrariums and dish gardens or as small potted plants.
Figure 6. Prayer plant sixteen weeks after potting a commercially rooted cutting into a 15.24 cm (6 inch) azalea pot (left); tissue-culture derived plantlet sixteen weeks after transfer into the greenhouse (right).
REFERENCES CITED


SECTION II. A COMPARISON OF THE LEAF ANATOMY OF MICROPROPAGATED
SHOOTS, ACCLIMATIZED PLANTLETS, AND GREENHOUSE-
GROWN PLANTS
ABSTRACT

This study compared the leaf anatomy of aseptically-cultured chrysanthemum (Chrysanthemum morifolium 'Bright Golden Anne') and carnation (Dianthus caryophyllus 'Scania') shoots, acclimatized plantlets, and greenhouse-grown cuttings. Leaves of aseptically-cultured chrysanthemum were found to be thinner than leaves produced during greenhouse acclimatization, and acclimatized leaves were thinner than leaves which formed in the greenhouse. Both the abaxial and adaxial epidermal layers in aseptically-cultured chrysanthemum leaves were thinner than epidermal layers in acclimatized leaves, and those observed in the epidermal layers in acclimatized leaves were thinner than greenhouse developed leaves. Carnation leaves produced in vitro were significantly thinner and formed thinner epidermal layers than leaves acclimatized or grown in the greenhouse environment. No differences in leaf and epidermal thicknesses were observed between greenhouse and acclimatized carnation leaves, however. Acclimatized chrysanthemum and carnation leaves had greater density of cells than aseptically-cultured leaves but lacked the density found in leaves which developed in the greenhouse. The largest numbers of chloroplasts per unit area were observed in greenhouse-grown chrysanthemum and carnation leaves while the lowest chloroplast density was observed in acclimatized leaves.
INTRODUCTION

Micropropagation has become an increasingly important means of rapidly propagating many plants. However, its commercial application for some species remains questionable. Difficulty re-establishing some plants into the greenhouse environment has been experienced. Only 50-70% of carnation (4, 9), 60% of blackberry (3), and 50-81% of rose (6, 8) plants survived the transfer to the greenhouse. Recent interest in finding a solution to this problem has led to an investigation of the effects of tissue culture on the leaf anatomy of aseptically-cultured plantlets.

Investigators have found significant differences in the anatomy of aseptically-cultured plantlets compared to acclimatized plantlets and greenhouse- or field-grown plants. Leaves of aseptically-cultured cauliflower (5), plum (2), and sweetgum (10) plantlets failed to develop a well-defined palisade mesophyll layer. Irregular parenchyma cells, interspersed by a large amount of air space, resembled spongy mesophyll (5). In 'Pixy' plum, small ovoid cells formed one poorly defined palisade layer (2). Abaxial epidermal cells in sweetgum were smaller and thinner walled than adaxial epidermal cells and cells in both epidermal layers were oval in contrast to the cubical shape observed in field-grown plants (10). Leaves of tissue culture derived sweetgum plantlets were thinner than leaves of acclimatized or field-grown plants (10).

Acclimatized plantlets were found to have an internal leaf anatomy somewhat more developed than aseptically-cultured plantlets. Mesophyll cells in the leaves of acclimatized cauliflower (5) and sweetgum (10) plantlets showed some degree of elongation but did not form a discernible
palisade layer. Leaves of 'Pixy' plum and sweetgum plantlets transferred to the greenhouse contained less mesophyll air space than in vitro plantlets, but lacked the density of cells found in leaves produced in the field (2, 10). All mesophyll cells in aseptically-cultured cauliflower and sweetgum plantlets, acclimatized sweetgum plants, and conventionally-grown cauliflower and sweetgum plants were found to contain chloroplasts (5, 10). Chloroplast division is controlled by irradiance levels (7).

The objective of this study was to compare the leaf anatomy of chrysanthemum and carnation micropropagated shoots, acclimatized plantlets, and greenhouse-grown cuttings.
MATERIALS AND METHODS

Aseptic culture Carnation (Dianthus caryophyllus 'Scania') and chrysanthemum (Chrysanthemum morifolium 'Bright Golden Anne') were multiplied and rooted in 25 x 150 mm culture tubes containing 15 ml Linsmaier-Skoog (LS) medium. For multiplication purposes, the medium was supplemented with 2.0 mg/liter kinetin and 0.1 mg/liter NAA. No supplementary hormones were added to promote root development. The pH of the medium was adjusted to 5.8 prior to adding 8 g/liter Difco® Bacto agar (6 g/liter for carnation) and autoclaving for 15 minutes at 121 C. Subcultured shoot tips were grown for 4 weeks in a Sherer TC-32 growth chamber under cool white fluorescent lights which provided 83 ± 11 μEm⁻²s⁻¹ photosynthetic photon flux density (PPFD) during a 16 hour daily photoperiod. The temperature in the growth chamber was maintained at 22 ± 1 C. The experimental design was completely randomized with 4 replicates consisting of 4 plants each. The newest fully expanded leaf was excised for anatomical observations.

Acclimatization procedures Following the removal of rooted carnation and chrysanthemum shoots from the in vitro environment, excess medium was washed off the roots with distilled water and they were planted individually into 5.7 X 3.2 X 5.7 cm cell packs containing a 1 perlite: 1 Hyphnum peatmoss (v/v) medium. The shoots were grown under an intermittent mist system which was activated by evaporation of moisture off a screen. Mist was applied every 15 - 17 minutes for 4 seconds on cloudy days and every 10 - 12 minutes for 10 seconds on sunny days. After 7 days under mist, the shoots were repotted into 5.7 cm pots containing a
1 soil: 1 *Hypnum* peatmoss: 3 perlite (v/v/v) medium and placed under 63% shade in the greenhouse. Irradiance levels of $152 \pm 14 \text{ uEm}^{-2}\text{s}^{-1}$ were recorded at 3:00 p.m. on sunny afternoons. Greenhouse temperatures were maintained at $23 \pm 5 \text{ C}$. The first leaf which unfurled and expanded, following transfer into the greenhouse, was excised from each plant.

**Greenhouse culture** Commercial carnation and chrysanthemum cuttings were potted into 10 cm plastic pots containing a 1 *Hypnum* peatmoss: 1 perlite: 1 soil (v/v/v) medium. The cuttings were placed into the greenhouse environment where they were allowed to grow for 2 months in full sunlight. Water soluble fertilizer (20N-20P-20K) was applied weekly at a rate of 416 mg/liter. Irradiance levels of $710 \pm 47 \text{ uEm}^{-2}\text{s}^{-1}$ were measured on sunny afternoons. A temperature of $23 \pm 5 \text{ C}$ was maintained. The newest fully expanded leaf was excised from each plant.

**Leaf anatomy** Excised carnation and chrysanthemum leaves were fixed in FAA (formalin-acetic-alcohol) for 24 hours, embedded in paraffin, sectioned 10 um thick, and stained with safranin and fast green (1). Leaf thickness was measured 5 times per carnation and chrysanthemum cross section with the aid of a light microscope. Chloroplasts were counted in 3 cells adjacent to the adaxial epidermis, the cell area was determined, and the number of chloroplasts per um$^2$ was calculated. Measurements of the depth of 3 abaxial and adaxial epidermal cells were made. The depth of 3 palisade cells was measured on cross sections of chrysanthemum leaves from the 3 environments. All measurements were made randomly; however, care was taken to avoid veins, trichomes, and stomata. Data for each species were separately subjected to an analysis of variance and least significant
differences (P = 0.05) were calculated. The amount of intercellular air space was observed visually in both chrysanthemum and carnation.
RESULTS

Differences in carnation and chrysanthemum leaf cross sections of aseptically-cultured plantlets, acclimatized plants, and greenhouse-grown cuttings were observed. Cross sections of leaves of aseptically-cultured chrysanthemum were found to be 165 µm thick, leaves produced during greenhouse acclimatization were 236 µm thick, and leaves which formed in the shaded greenhouse were 250 µm thick (Table 1). Significant differences in the depth of both the abaxial and adaxial epidermal layers were observed among leaves formed in the three cultural treatments. No differences in leaf thickness or the depth of the epidermal layers were noted between acclimatized and greenhouse-developed carnation leaves (Table 1). Aseptically-cultured carnation shoots, however, formed leaves one-fourth as thick and epidermal layers one-half as thick as acclimatized and greenhouse leaves. Epidermal cells in aseptically-cultured carnation and chrysanthemum leaves lacked the cubical or rectangular shapes found in greenhouse leaves (Figures 1 and 2).

A poorly defined palisade layer was observed in aseptically-cultured chrysanthemum leaves (Figure 1A). Cells adjacent to the adaxial epidermis showed some degree of elongation but the length of these cells (35 µm) was significantly less than palisade cells which developed in leaves produced during acclimatization (61 µm) and in the greenhouse (61 µm). Palisade cells in acclimatized leaves were similar in size and shape to palisade cells in greenhouse leaves (Figures 1B and 1C). However, the palisade layers in acclimatized leaves lacked the density of the palisade layers in greenhouse leaves.
Table 1. A comparison of the leaf anatomy of aseptically-cultured chrysanthemum and carnation plantlets to acclimatized plants and greenhouse-grown cuttings.\(^z\)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Cultural treatment(^Y)</th>
<th>Leaf thickness (um)</th>
<th>Depth of epidermis (um)</th>
<th>Depth of palisade layer (um)</th>
<th>Chloroplasts per um(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Abaxial</td>
<td>Adaxial</td>
<td></td>
</tr>
<tr>
<td>Chrysanthemum</td>
<td>ghs</td>
<td>250</td>
<td>21</td>
<td>28</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>accl</td>
<td>236</td>
<td>16</td>
<td>24</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>asep</td>
<td>165</td>
<td>14</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>LSD 5%</td>
<td>18</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Carnation</td>
<td>ghs</td>
<td>410</td>
<td>29</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>accl</td>
<td>400</td>
<td>32</td>
<td>32</td>
<td>-</td>
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<td></td>
<td>asep</td>
<td>119</td>
<td>14</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LSD 5%</td>
<td>34</td>
<td>3</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^z\)Analysis of the means of 16 individual leaves.

\(^Y\)Cultural treatments include greenhouse-grown (ghs), greenhouse acclimatized (accl), and aseptically-cultured (asep) plants.
Figure 1. Cross sections of chrysanthemum leaves excised from A) aseptically-cultured plantlets, B) acclimatized plants, and C) greenhouse-grown cuttings.
Figure 2. Cross sections of carnation leaves excised from A) aseptically-cultured plantlets, B) acclimatized plants, and C) greenhouse-grown cuttings.
Figure 2. Cross sections of carnation leaves excised from A) aseptically-cultured plantlets, B) acclimatized plants, and C) greenhouse-grown cuttings.
Carnation and chrysanthemum leaves produced in vitro contained large intercellular spaces. Acclimatized leaves contained less mesophyll air space than aseptically-cultures leaves but more than leaves which developed in the greenhouse (Figures 1 and 2).

Greater chloroplast density was observed in greenhouse-developed chrysanthemum leaves (0.030 chloroplasts per $\mu m^2$) than in acclimatized leaves (0.018 chloroplasts per $\mu m^2$) and aseptically-cultured leaves (0.024 chloroplasts per $\mu m^2$) (Table 1). Chloroplast densities showed the same general trend in carnation leaves as they did in chrysanthemum leaves. The greatest density was observed in greenhouse-developed carnation leaves (0.044 chloroplasts per $\mu m^2$), the lowest density in acclimatized leaves (0.028 chloroplasts per $\mu m^2$), and an intermediate density in aseptically-cultured leaves (0.037 chloroplasts per $\mu m^2$).
DISCUSSION

The leaf anatomies of micropropagated shoots, acclimatized plantlets, and greenhouse-grown cuttings of chrysanthemum and carnation were found to be different. In chrysanthemum, leaf thickness and the depth of the epidermal layers differed significantly among leaves which developed in the three environments. Aseptically-cultured chrysanthemum leaves were found to be thinner than leaves produced during greenhouse acclimatization, and acclimatized leaves were thinner than leaves which formed in the shaded greenhouse. Aseptically-cultured carnation shoots formed the thinnest leaves. These findings agree with earlier reports which stated that aseptically-cultured sweetgum plantlets developed leaves which were thinner than leaves which developed in the field or during acclimatization (10).

Sweetgum leaves produced in vitro were found to develop smaller abaxial epidermal cells than adaxial cells and cells in both epidermal layers lacked the cubical shape observed in leaves which matured in the field (10). In chrysanthemum and carnation leaves excised from aseptically-cultured plantlets, both the abaxial and adaxial epidermal layers were thinner than epidermal layers in acclimatized and greenhouse-developed leaves. Epidermal cells in aseptically-cultured carnation and chrysanthemum leaves lacked the cubical or rectangular shapes found in greenhouse leaves.

Chrysanthemum leaves produced in vitro failed to develop a clearly defined palisade layer but cells adjacent to the adaxial epidermis did show some degree of elongation. Similar findings have been reported in
cauliflower (5) and sweetgum (10) plantlets. The palisade layers formed in acclimatized chrysanthemum leaves were not as compact as those formed in greenhouse-developed leaves. Cells were similar in size and shape but more intercellular air space surrounded the palisade cells. Acclimatized chrysanthemum leaves appeared to be intermediate in the degree of development of the internal leaf structure. This is similar to the findings which show that leaves of acclimatized 'Pixy' plum and sweetgum plantlets contain less mesophyll air space than leaves produced in vitro but lacked the cellular density found in leaves which developed in the field (2).

Both the palisade and spongy mesophyll cells in cross sections of chrysanthemum and carnation leaves cultured in the three environments contained chloroplasts. Similar findings have been reported in cauliflower (5) and sweetgum (10). The number of chloroplasts per unit area in chrysanthemum and carnation leaves developed in the three environments varied significantly. Chloroplast division is controlled by irradiance levels (7). In both chrysanthemum and carnation, the greatest chloroplast density within cells was observed in leaves developed in the greenhouse where the highest PPFD levels were recorded (710 uEm⁻²s⁻¹). Chrysanthemum and carnation leaves acclimatized in the shaded greenhouse (PPFD levels of 152 uEm⁻²s⁻¹) contained the lowest density of chloroplasts. The chloroplast densities given in this paper do not reflect the number of chloroplasts per unit area of leaf cross section because the amount of intercellular air space varied greatly among leaves cultured in the three environments.
REFERENCES CITED


SECTION III. THE EFFECTS OF VARIOUS PHOTOSYNTHETIC PHOTON
FLUX DENSITIES ON THE LEAF ANATOMY AND
CHLOROPHYLL CONTENT OF TISSUE-CULTURED PLANTS
ABSTRACT

In vitro cultures of spathiphyllum (Spathiphyllum wallisii), maranta (Maranta leuconeura 'Kerchoviana'), carnation (Dianthus caryophyllus 'Scania'), and chrysanthemum (Chrysanthemum morifolium 'Bright Golden Anne') were incubated under three photosynthetic photon flux densities (PPFD)(52, 114, and 185 uEm⁻²s⁻¹) during growth stage III to determine if varying irradiance levels within the growth chamber environment affected leaf structure or chlorophyll content. The chlorophyll a content in carnation leaves increased as the PPFD level increased from 52 to 185 uEm⁻²s⁻¹ while in maranta the chlorophyll a and chlorophyll b levels decreased. The chlorophyll contents of chrysanthemum and spathiphyllum leaves developed under the three PPFD levels did not vary significantly. No significant differences in the number of chloroplasts per unit area were observed in any of the four species examined. Leaf cross sections of maranta grown at 185 uEm⁻²s⁻¹ exhibited a reduction in mesophyll air space compared to leaves grown under the lower PPFD levels. Spathiphyllum leaves cultured under 185 uEm⁻²s⁻¹ were significantly thinner than leaves which developed under 52 uEm⁻²s⁻¹ while maranta leaves cultured at the higher PPFD levels were significantly thicker.
INTRODUCTION

The problem of transferring tissue-cultured plantlets to a greenhouse environment has been documented (5, 9, 18, 23, 24). The involvement of water in this relationship has been studied (2, 3, 9, 12, 13, 14, 24, 25), but the influence of light intensity, particularly as a pretreatment, has not been investigated thoroughly. One approach to evaluating this factor would be to study its effect on leaf anatomy and chlorophyll content.

Leaves which develop in low irradiance levels have an increase in leaf size, decrease in leaf thickness (8), increase in chlorophyll concentrations (6, 7), and an altered internal leaf structure compared to leaves which developed under high irradiance levels (8). Palisade tissue is absent or poorly developed in shade leaves and the loosely organized mesophyll cells are interspersed by large air spaces (8).

Irradiance levels also influence chloroplast development and orientation (26). Under low irradiance levels chloroplasts are distributed horizontally along the adaxial and abaxial cell walls to allow for maximum PPFD interception (8, 26). Chloroplasts are oriented vertically along lateral walls in sun leaves (8, 26). Chloroplast division is controlled by irradiance levels and wavelength (22).

Modifications of the leaf anatomy of aseptically-cultured plantlets have been noted, such as incomplete development of the palisade mesophyll and extensive intercellular space (4, 15, 25). Palisade parenchyma cells in 'Pixy' plum plantlets were shorter than those in acclimatized or field-grown plants (4). In sweetgum plantlets, chloroplasts were flattened and lacked organization of the internal membranes (25). Sweetgum plantlets
cultured in vitro formed thinner leaves than acclimatized or field-grown plants (25). Leaves of stage III cauliflower plantlets were found to have reduced levels of chlorophyll (16).

Results of previous studies on the leaf anatomy of aseptically-cultured plants describes an internal structure reminiscent of that found in shade leaves. The objective of this study was to determine if various irradiance levels within the growth chamber environment had an effect on the internal leaf anatomy or chlorophyll content of leaves developed during stage III.
MATERIALS AND METHODS

Aseptic culture Carnation (Dianthus caryophyllus 'Scania'), chrysanthemum (Chrysanthemum morifolium 'Bright Golden Anne'), maranta (Maranta leuconeura 'Kerchoviana'), and spathiphyllum (Spathiphyllum wallisii) shoots were multiplied in 25 X 150 mm culture tubes containing 15 ml of basal medium. Axillary maranta and spathiphyllum shoots were produced on Linsmaier-Skoog (LS) medium supplemented with 0.2 mg/liter BA and Murashige-Skoog (MS) medium containing 1.0 mg/liter kinetin, respectively. Chrysanthemum and carnation shoots were multiplied on LS medium supplemented with 2.0 mg/liter kinetin and 0.1 mg/liter NAA. The pH of the medium was adjusted to 5.8 prior to adding 8 g/liter (6 g/liter for carnation) of DifcoR Bacto agar and autoclaving for 15 minutes at 121 C.

Irradiance treatments Spathiphyllum and maranta shoots and carnation and chrysanthemum shoot tips were subcultured into 25 X 150 mm culture tubes containing 15 ml of full-strength LS medium (MS medium for S. wallisii). No supplementary growth regulators were added. Subcultured shoots and shoot tips were grown at 26 ± 1 C for four weeks in Sherer TC-32 growth chambers equipped with cool white fluorescent lights. Plants were grown under photosynthetic photon flux densities (PPFD) of 52 ± 6, 114 ± 7, and 185 ± 7 uEm\(^{-2}\)s\(^{-1}\) for 16 hours daily. PPFD levels were measured at plant height with a LI-185A quantum radiometer (LI-COR Inc., Lincoln, NE). Irradiance treatments were replicated 4 times in a completely randomized design. The data for each species were separately subjected to an analysis of variance and least significant differences
(LSD) were calculated at the 5% level.

**Leaf anatomy**  The newest fully expanded leaf of each shoot was fixed in FAA (formalin-acetic-alcohol). Paraffin-embedded cross sections, 10μm thick, were stained with safranin and fast green (1) and viewed with a light microscope. Leaf thickness was measured in 5 randomly chosen microscopic fields for each slide. Care was taken to avoid veins and leaf margins. Chloroplast counts were made on 3 randomly selected mesophyll cells adjacent to the adaxial epidermis. The area of each mesophyll cell was calculated and the number of chloroplasts per um² was determined. Chloroplast distribution and the amount of mesophyll air space were also noted. Two leaves were sampled per replication.

**Leaf chlorophyll content**  Either the most recently expanded leaf or whorl of leaves (carnation) from light-treated, aseptically-cultured shoots was excised and weighed. The sample was ground in a mortar in 5 ml of 70% (v/v) methanol, and the homogenate was forced through a Millipore Millex®-SR 0.50 um filter into a C₁₈ Sep-Pak™ reverse phase cartridge. Pigments were eluted from the Sep-Pak™ cartridge with 100% methanol as previously described (11). Aliquots of the eluted pigments were analyzed by high performance liquid chromatography (HPLC). A 40 minute run time using 50% solvent A (80% methanol) and 50% solvent B (ethyl acetate) gave baseline separation of chlorophylls a and b and complete elution of other pigments from the column. Leaf chlorophyll concentrations were calculated on a mg chl/g fresh weight basis, and the chlorophyll a to b ratio was determined. Two leaves or leaf whorls were analyzed per replication.
RESULTS

Differences in the leaf anatomy and chlorophyll content of aseptically-cultured plantlets rooted in vitro under 3 PPFD levels have been observed. Carnation leaves developed under 185 μEm⁻²s⁻¹ contained significantly more chlorophyll a than leaves that developed under 52 μEm⁻²s⁻¹ (Table 1). However, irradiance levels had no significant effect on the chlorophyll b content of carnation leaves. Maranta leaves formed under 185 μEm⁻²s⁻¹ contained significantly less chlorophyll a and chlorophyll b than leaves formed under 52 μEm⁻²s⁻¹. The chlorophyll a and chlorophyll b contents of chrysanthemum and spathiphyllum leaves did not vary significantly. The chlorophyll a to chlorophyll b ratio was 2.88 to 3.23 in all species.

No significant differences in the chloroplast densities were observed in the 4 species examined. Carnation and maranta leaves contained 0.036 - 0.046 chloroplasts per um² and chrysanthemum and spathiphyllum leaves contained 0.015 - 0.019 chloroplasts per um².

In the mesophyll cells adjacent to the adaxial epidermis, chloroplasts were found to be tightly aligned against the lower cell walls in spathiphyllum leaves cultured under 185 μEm⁻²s⁻¹. In maranta cultured under PPFD levels of 185 μEm⁻²s⁻¹, the chloroplasts were clustered in the middle of the lower portion of the cells. Chloroplasts were scattered along lateral walls in maranta and spathiphyllum cultured under the lower irradiance levels and in carnation and chrysanthemum cultured under the three irradiance levels.

Maranta leaf thickness increased significantly from 98 to 109 um as
Table 1. The effects of irradiance levels in vitro on the chlorophyll content and thickness of leaves and number of chloroplasts per unit area.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Irradiance levels (uEm^{-2}s^{-1})</th>
<th>Chlorophyll a (mg/g fresh wt)</th>
<th>Chlorophyll b (mg/g fresh wt)</th>
<th>Chlorophyll a:b</th>
<th>Chlorophyll thickness (um)</th>
<th>Chloroplasts per um^2</th>
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<tr>
<td>Chrysanthemum</td>
<td>185</td>
<td>0.72</td>
<td>0.23</td>
<td>3.21</td>
<td>151</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>114</td>
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<td>0.24</td>
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<td></td>
<td>52</td>
<td>0.74</td>
<td>0.23</td>
<td>3.23</td>
<td>148</td>
<td>0.015</td>
</tr>
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<td></td>
<td>LSD 5%</td>
<td>0.20</td>
<td>0.06</td>
<td>0.24</td>
<td>25</td>
<td>0.006</td>
</tr>
<tr>
<td>Carnation</td>
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<td>1.24</td>
<td>0.41</td>
<td>3.06</td>
<td>130</td>
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</tr>
<tr>
<td></td>
<td>114</td>
<td>1.17</td>
<td>0.39</td>
<td>3.01</td>
<td>143</td>
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<tr>
<td></td>
<td>52</td>
<td>0.96</td>
<td>0.33</td>
<td>2.96</td>
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<td>Maranta</td>
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<td>3.18</td>
<td>109</td>
<td>0.046</td>
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<td></td>
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<td>98</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>LSD 5%</td>
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<td>0.27</td>
<td>10</td>
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<tr>
<td>Spathiphyllum</td>
<td>185</td>
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<td></td>
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<tr>
<td></td>
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<tr>
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<td>0.11</td>
<td>0.31</td>
<td>20</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Analysis of the means of 8 individual leaves.*
the PPFD levels increased from 52 to 185 uEm\(^{-2}\)s\(^{-1}\). Leaf thickness in spathiphyllum decreased from 141 to 112 um as the PPFD levels increased to 185 uEm\(^{-2}\)s\(^{-1}\). No significant changes in the thickness of chrysanthemum and carnation leaves were observed.

Leaf cross sections of maranta grown at 185 uEm\(^{-2}\)s\(^{-1}\) exhibited a decrease in mesophyll air space compared to leaves grown at lower PPFD levels. The volume of intercellular air space in chrysanthemum, carnation, and spathiphyllum leaves was not influenced by the three irradiance levels.
DISCUSSION

The results of this study indicate that various PPFD levels in vitro have an effect on the leaf anatomy and chlorophyll content of aseptically-cultured plantlets. Although the chlorophyll \( a \) and \( b \) concentrations in chrysanthemum leaves were not affected by the irradiance levels, these concentrations in carnation increased with an increase in PPFD levels. Both maranta and spathiphyllum leaves contained an increasing amount of chlorophyll with decreasing PPFD levels. Similar results have been observed in *Ficus benjamina* L. leaves acclimatized under increasing levels of shade (6, 7). The chlorophyll \( a \) to chlorophyll \( b \) ratios were within the expected range (22).

Chlorophyll is distributed within the internal chloroplast membranes, the grana (21). Irradiance conditions which stimulate chlorophyll synthesis also induce chloroplast division. The synthesis of photosynthetic chloroplast membranes is dependent upon chlorophyll synthesis, and photosynthetic activity parallels the increase in chlorophyll concentrations and the synthesis of chloroplast membranes (17). Therefore, the number of chloroplasts per unit area might be expected to parallel the increases or decreases in chlorophyll concentrations which were observed in carnation and maranta. However, no significant differences in the number of chloroplasts per unit area were observed in any of the 4 species examined.

Irradiance levels have an effect on chloroplast distribution. In shade leaves, leaves expanded under low irradiance levels, chloroplasts are arranged along the adaxial and abaxial cell walls (8, 26). This
distribution pattern facilitates maximum absorption of the light entering the cell. Chloroplasts in sun leaves are positioned along vertical walls where they shade one another to prevent damage from excessive PPFD levels (8, 26). In carnation and chrysanthemum leaves cultured under the 3 irradiance levels, chloroplasts were found to be scattered along the lateral walls. This chloroplast arrangement resembled the distribution described in shade leaves where maximum interception of PPFD is of primary concern. Both carnation and chrysanthemum require very high irradiance levels for strong growth and good production (19). Therefore, the chloroplast distribution for these 2 species which allowed maximum PPFD absorption would be expected under the irradiance levels employed in this experiment.

Similar distribution patterns were observed in spathiphyllum and maranta grown under the 2 lowest irradiance levels. However, the chloroplasts in maranta cultured under 185 μEm⁻²s⁻¹ were found to be packed tightly into the middle of the mesophyll cells. This chloroplast distribution resembles the distribution noted in sun leaves, where the chloroplasts protect one another from excessive irradiance levels. Maranta has a very low light requirement (20); therefore, this chloroplast distribution would be expected at irradiance levels high enough to damage the photosynthetic mechanism.

The 3 irradiance levels used in this experiment had no effect on the thickness of chrysanthemum and carnation leaves. The thickness of spathiphyllum leaves decreased with increasing irradiance levels. Maranta leaves developed under 185 μEm⁻²s⁻¹ were significantly thicker and
contained less mesophyll air space than leaves which developed under the lower irradiance levels. Sun leaves are thicker and contain less mesophyll air space than shade leaves (8). Therefore, the increase in leaf thickness and the decrease in air space observed in maranta cultured under increasing PPFD levels were expected. The decrease in leaf thickness observed in spathiphyllum was contrary to the expected results.

The effects of the 3 irradiance levels on maranta leaf thickness and anatomy, chloroplast distribution, and chlorophyll concentrations indicate that the optimal irradiance level for maranta cultured in vitro is below 114 uEm⁻²s⁻¹.
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SECTION IV. THE EFFECTS OF VARIOUS PHOTOSYNTHETIC PHOTON FLUX DENSITIES IN VITRO AND SUBSEQUENT ACCLIMATIZATION TECHNIQUES ON THE SURVIVABILITY OF TISSUE-CULTURED PLANTS
ABSTRACT

Maranta (Maranta leuconeura 'Kerchoviana') and chrysanthemum (Chrysanthemum morifolium 'Bright Golden Anne') shoots were rooted in vitro under 3 irradiance levels (58, 112, and 186 uEm⁻²s⁻¹). Chrysanthemum shoots cultured under 186 uEm⁻²s⁻¹ were found to be significantly shorter than shoots rooted under irradiance levels of 58 or 112 uEm⁻²s⁻¹. The various irradiance levels had no effect on maranta shoot length or chrysanthemum root development. However, extensive lateral root development was observed on maranta plantlets cultured under 186 uEm⁻²s⁻¹. Leaf production was not influenced by irradiance levels used during stage III. Following a 4 week rooting period, plantlets were acclimatized in 1 of 3 environments: a shaded greenhouse, a humidity tent, or under intermittent mist. Chrysanthemum plantlets acclimatized under intermittent mist and in the shaded greenhouse developed 2.6 new leaves per plantlet during the acclimatization period, while plantlets acclimatized in the humidity tent formed 1.0 new leaf. Larger increases in chrysanthemum shoot length were observed on plantlets acclimatized under intermittent mist and in the shaded greenhouse. Acclimatization techniques had no effect on the growth rate of maranta plants. Significantly more maranta leaf injury was sustained on plantlets acclimatized in the humidity tent.
INTRODUCTION

The successful transfer of aseptically-cultured plantlets into the greenhouse environment continues to be a problem for some species. Poor survival rates of carnation (6, 19), blackberry (4), and rose (13, 17) have been reported. Recently, investigators have studied epicuticular wax formation (7, 8, 9, 19, 20), stomate structure (1, 20), frequencies (3), and functioning (1, 2), and transpiration rates (3, 7, 9) following the transfer of plantlets to the greenhouse. Results of these studies indicate plant losses are a result of excessive water loss due to a lack of epicuticular wax formation (6, 7, 9, 19) and malfunctioning of the stomatal closure mechanism (2, 7, 19). In 1978, it was reported that cauliflower plantlets had low photosynthetic rates and reduced levels of chlorophyll at the time of transplanting (11). These factors were thought to contribute to their vulnerability.

Several methods of acclimatizing tissue-cultured plantlets have been developed. Humidity tents and/or intermittent mist systems are used to convert micropropagated shoots and plantlets into autotrophic plants, able to withstand the greenhouse environment (5, 12, 14, 18).

The objective of this study was to determine if various irradiances in vitro and subsequent acclimatization techniques affected the relative growth rates and survivability of plants following transfer to the greenhouse environment.
MATERIALS AND METHODS

Aseptic culture  Chrysanthemum (C. morifolium 'Bright Golden Anne') and maranta (M. leuconeura 'Kerchoviana') shoots were multiplied on 15 ml of Linsmaier-Skoog (LS) medium supplemented with 2.0 mg/liter kinetin plus 0.1 mg/liter NAA and 0.2 mg/liter BA, respectively. The pH of the medium was adjusted to 5.8 prior to adding 8 g/liter Difco® Bacto agar, pipetting into 25 X 150 mm culture tubes, and autoclaving for 15 minutes at 121 C.

Irradiance treatments  Chrysanthemum and maranta shoot tips, each having equal length and vigor, were subcultured onto 15 ml of LS medium in 25 X 150 mm culture tubes to promote root development. Fifteen chrysanthemum and maranta shoots were randomly assigned to an irradiance treatment in Sherer TC-32 growth chambers for 4 weeks. Photosynthetic photon flux densities (PPFD) of 58 ± 3, 112 ± 6, and 186 ± 5 uEm^{-2}s^{-1} were supplied by cool white fluorescent lights during a 16 hour photoperiod. PPFD levels were measured at plant height with a LI-185A quantum radiometer (LI-COR Inc., Lincoln, NE). A temperature of 24 ± 1 C was maintained. The relative growth rates of chrysanthemum and maranta shoots during stage III were examined.

Acclimatization treatments  After rooting in vitro, aseptically-produced chrysanthemum and maranta plantlets were transferred to acclimatization environments in the greenhouse. Shoot and root lengths were measured, leaves counted, and the degree of lateral root formation was noted. Roots were washed with distilled water to remove excess medium prior to planting in plastic cell packs (5.7 X 3.2 X 5.7 cm) containing a
1 Hypnum: 3 perlite (v/v) medium. All plants were placed under an intermittent mist system for 4 days. Then, 5 plants of each species grown under each irradiance treatment were placed into a shaded greenhouse (63% shade cloth) or a humidity tent for 7 days. The remaining 5 plants stayed in the mist for an additional 5 days before they were moved into the shaded greenhouse. After 1 week, plants in the humidity tent were moved to the shaded greenhouse.

The intermittent mist system was controlled by evaporation of moisture off a counterbalance screen. On sunny days, mist was applied every 10 - 12 minutes for 10 seconds. The mist interval increased to every 15 - 17 minutes for 4 seconds on overcast and rainy days. Irradiance levels were not measured under the mist. The temperature at leaf height was 21 ± 0.5°C at mid-afternoon.

The humidity tent was constructed of polyethylene covered with 30% shade cloth. A further reduction in PPFD levels resulted from shading by structural members of the greenhouse due to the low angle of the sun. Irradiance levels of 85 ± 12 μEm⁻²s⁻¹ were measured on sunny afternoons with a LI-185A quantum radiometer (LI-COR Inc., Lincoln, NE). Adequate ventilation was provided within the tent to prevent excessive heat gain. Air temperatures of 23 ± 1°C were recorded. High humidity levels were maintained by watering the concrete floor regularly and by atomizing water into the air approximately once each hour from 11:00 a.m. to 3:00 p.m.

Plants placed into the shaded greenhouse received 141 ± 12 μEm⁻²s⁻¹ PPFD at 3:00 p.m. A temperature of 23 ± 4.7°C was maintained.

After a 2 week acclimatization period, plants were removed from the
cell packs and excess rooting medium was washed off the roots with tap water. Shoot and root lengths were measured. Leaves were counted and the number of injured leaves were noted. Leaves showing some degree of chlorosis or necrosis were considered to be injured. The degree of lateral root formation was assessed visually.

**Experimental design**  
A split-plot experimental design was used. Chrysanthemum and maranta plants were randomly assigned to 1 of 3 irradiance levels. After a 4 week rooting period, 5 chrysanthemum and 5 maranta plants rooted under each irradiance level were randomly assigned to each of 3 acclimatization treatments.

**Statistical analysis**  
Changes in shoot and root length during the rooting and acclimatization stages were calculated. The increases in the number of leaves produced during the rooting and acclimatization periods were recorded. The degree of lateral root development and axillary shoot formation was observed for maranta. The data were subjected to an analysis of variance and the least significant differences (LSD) were calculated at the 5% level. Data were analyzed separately for each species.
RESULTS

Chrysanthemum shoots cultured under an irradiance level of 186 uEm$^{-2}$s$^{-1}$ were significantly shorter than shoots cultured under 58 or 112 uEm$^{-2}$s$^{-1}$ (Table 1). Varying irradiance levels had no significant effect on the increase in length of maranta shoots. Also, the 3 irradiance levels had no effect on the number of new leaves produced by either species. Maranta plantlets rooted under 186 uEm$^{-2}$s$^{-1}$ were observed to develop shorter, more branched root systems while roots which formed on plantlets cultured under 58 and 112 uEm$^{-2}$s$^{-1}$ were longer and produced fewer lateral roots (Figure 1). Irradiance levels in vitro did not noticeably alter the root morphology of chrysanthemum plantlets.

The acclimatization techniques had differential effects on chrysanthemum shoot and root elongation and leaf development but not on those of maranta. Chrysanthemum plantlets acclimatized in a humidity tent expanded significantly fewer leaves per plantlet than those acclimatized under the intermittent mist or in the greenhouse (Table 2). The same was true for the increase in shoot length. Significantly different increases in chrysanthemum root growth occurred in plantlets acclimatized in the 3 different environments. More root development was observed in plantlets acclimatized under the intermittent mist than was measured in the other 2 treatments. The least amount of root growth occurred in the greenhouse.

The incidence of leaf injury which occurred during acclimatization was observed. Maranta plantlets acclimatized in the humidity tent showed the highest incidence of leaf injury (Table 3). Significantly less leaf injury was sustained by plantlets acclimatized in the greenhouse.
Table 1. Relative growth rates of chrysanthemum shoots rooted in *vitro* under three irradiance levels.

<table>
<thead>
<tr>
<th>Irradiance level (μE m⁻² s⁻¹)</th>
<th>Shoot length(^2) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>185</td>
<td>28.3</td>
</tr>
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<td>114</td>
<td>38.1</td>
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<td>52</td>
<td>40.9</td>
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<td>LSD 5%</td>
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</tbody>
</table>

\(^2\)Mean of 15 individual plants cultured *in vitro* for 4 weeks. Shoot lengths were equivalent at beginning of experiment.
Figure 1. Maranta plantlets cultured in vitro under A) 58 uEm$^{-2} \text{s}^{-1}$, B) 112 uEm$^{-2} \text{s}^{-1}$, and C) 186 uEm$^{-2} \text{s}^{-1}$ PPFD.
Table 2. The effects of three acclimatization techniques on chrysanthemum growth rates.\textsuperscript{z}

<table>
<thead>
<tr>
<th>Acclimatization technique</th>
<th>No. of new leaves</th>
<th>Increase in length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>shoots</td>
</tr>
<tr>
<td>Humidity tent</td>
<td>2.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Intermittent mist</td>
<td>2.6</td>
<td>21.5</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>2.6</td>
<td>19.0</td>
</tr>
<tr>
<td>LSD 5%</td>
<td>0.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\textsuperscript{z}Mean of 15 individual plants.
Table 3. The effects of three acclimatization techniques on maranta leaf injury incurred during acclimatization.

<table>
<thead>
<tr>
<th>Acclimatization Technique</th>
<th>Number of Leaves Injured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humidity tent</td>
<td>2.7</td>
</tr>
<tr>
<td>Intermittent mist</td>
<td>1.6</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>1.0</td>
</tr>
<tr>
<td>LSD 5%</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Means of 15 individual plants.*
Neither the irradiance level employed in vitro nor the acclimatization technique used significantly influenced lateral shoot development in maranta.

No significant interactions between the irradiance levels used during stage III and the subsequent acclimatization techniques were observed.
DISCUSSION

Increasing irradiance levels from 58 to 186 uEm\(^{-2}\)s\(^{-1}\) in vitro affected chrysanthemum shoot elongation. Chrysanthemum shoots cultured under the 2 lowest irradiance levels were significantly longer than shoots cultured under the highest level. The increases in shoot length were due to internodal elongation induced by low irradiance levels. Chrysanthemum plantlets are most productive under high irradiance levels, and under low irradiance levels the plantlets become elongated and develop weak stems (15). This would indicate that only the highest irradiance level used in this experiment was sufficient to sustain regular growth of chrysanthemum plantlets in vitro.

The 3 irradiance levels used during stage III did not have a significant effect on maranta shoot growth or the production of new leaves. However, maranta plantlets cultured under 186 uEm\(^{-2}\)s\(^{-1}\) developed extensive root systems. Plantlets cultured under 58 and 112 uEm\(^{-2}\)s\(^{-1}\) did develop numerous lateral roots, but the extent of the lateral root formation was considerably less than that observed at the highest irradiance level. This difference in root development did not seem to influence acclimatization success, however.

Different acclimatization environments affected chrysanthemum shoot and root elongation and leaf development, and plants acclimatized under intermittent mist exhibited the largest increases in shoot and root elongation. Root length increased 47.2 mm, shoots increased by 21.5 mm, and 2.6 new leaves expanded per plant. A reduction in chrysanthemum leaf temperatures, due to evaporation of water from the leaf surface, was
thought to decrease the rate of photorespiration which allowed a greater quantity of the photosynthates to be available for growth. Biological reactions such as photosynthesis, photorespiration, and respiration are extremely sensitive to temperature. At lower temperatures, respiratory rates are reduced and the amount of photosynthates utilized for plant growth usually increases (16).

The mean shoot length increases of chrysanthemum plantlets acclimatized in the greenhouse were not significantly different from the results observed in chrysanthemum plantlets acclimatized under intermittent mist. However, root length increased significantly less in the greenhouse than in either of the other 2 acclimatization environments. Chrysanthemum plantlets acclimatized in the humidity tent, however, attained the least amount of increase in shoot length and leaf development. Plantlets acclimatized in the humidity tent routinely wilted during the middle portion of the day even though temperatures within the tent were not excessive, humidity levels were very high, and the soil medium contained adequate moisture. This should explain the lower increase of shoot elongation. Under the conditions of this experiment, chrysanthemum plants acclimatized under intermittent mist showed the largest increases in overall growth. Following acclimatization under mist, the plantlets were transferred into the greenhouse without difficulty.

The elongation of shoots and roots and the development of new leaves by maranta plants during acclimatization were not significantly affected by the techniques employed, but these techniques had a significant effect
on the amount of leaf injury sustained during the acclimatization period. Plantlets acclimatized in the humidity tent sustained the greatest amount of damage, possibly as a result of the daily wilting. Under the mist, leaf damage occurred as a rot which could be related to the water film present on the leaves. Leaves in the shaded greenhouse environment sustained the least damage. These results suggest that maranta plants are better adapted to the lower humidity levels of the shaded greenhouse. Because maranta is a relatively slow growing foliage plant, significant increases in shoot and root length and leaf development were not observed during the 4 week irradiance treatments or the 2 week acclimatization treatments.
REFERENCES CITED


OVERALL SUMMARY AND CONCLUSIONS

*Maranta leuconeura* 'Kerchoviana' (maranta) can be micropropagated successfully. Excised lateral buds (2 - 5 mm) and shoots at the 2-leaf stage cultured on a full-strength Linsmaier-Skoog (LS) medium supplemented with 0.2 mg/liter BA developed 3 - 4 and 5 - 6 lateral shoots, respectively, in 12 weeks. Lateral shoots developed roots in 3 - 4 weeks on a full-strength (LS) medium containing no supplementary hormones. After root development, the shoots were easily established in a shaded greenhouse environment. *Maranta* plants derived from tissue culture are compact and attractive.

Micropropagation had an effect on the internal leaf structures of *Chrysanthemum morifolium* 'Bright Golden Anne' (chrysanthemum) and *Dianthus caryophyllus* 'Scania' (carnation). The leaf anatomies of micropropagated shoots, acclimatized plantlets, and greenhouse-grown cuttings of chrysanthemum and carnation were found to be significantly different. Plantlets cultured in vitro produced leaves similar in structure to shade leaves. The leaves were thinner, developed thinner adaxial and abaxial epidermal layers, and contained more mesophyll air space than acclimatized or greenhouse-developed leaves. Aseptically-cultured leaves contained fewer chloroplasts per um² of cell space than leaves which developed in the greenhouse. The leaf structure of acclimatized leaves was found to be more developed than aseptically-cultured leaves but less developed than the greenhouse (sun) leaves.

Various photosynthetic photon flux densities (PPFD) during stage III were found to have differential effects on the leaf anatomy and
chlorophyll contents of carnation, chrysanthemum, maranta, and spathiphyllum. The 3 irradiance levels used in this experiment (52, 114, and 185 uE·m⁻²·s⁻¹) had significant effects on maranta leaf thickness and anatomy, chloroplast distribution, and chlorophyll concentrations. These results indicate that the optimal irradiance level for maranta cultured in vitro is below 114 uE·m⁻²·s⁻¹.

The spathiphyllum results in this study are conflicting based on the results of maranta, a shade plant, and of chrysanthemum and carnation, sun plants. Spathiphyllum is also a shade plant, but the irradiance levels were found to have no effect on its chlorophyll content and volume of mesophyll air space, similar to the findings for chrysanthemum and carnation leaves. Chloroplast distribution at the highest irradiance level, however, was similar to that observed in maranta leaves. The increase in leaf thickness with decreasing irradiance level was contrary to the pattern generally observed in sun and shade leaves. These findings indicate that the anatomical reaction of spathiphyllum grown in vitro to a change in irradiance level is not predictable.

The 3 irradiance levels (58, 112, and 186 uE·m⁻²·s⁻¹) had a differential effect on stem elongation of chrysanthemum shoots with the 2 lower levels producing more elongation than should be expected. This indicates an irradiance level in excess of 112 uE·m⁻²·s⁻¹ is necessary for normal chrysanthemum growth in vitro. The root system of maranta was more highly branched at the highest irradiance level as compared to the other levels.

These results indicate irradiance levels in vitro can be altered
sufficiently to have an effect on plantlet leaf anatomy, chlorophyll content, stem elongation, and root morphology. Higher irradiance levels are required by some species than others to support normal growth, and the optimal level must be determined for the individual species being cultured.

Various acclimatization techniques can be employed to prepare tissue culture derived plantlets for transfer into the greenhouse environment. The results of this study indicate that the appropriate acclimatization technique for a specific species must be determined. Greater increases in shoot and root lengths and leaf development were observed in chrysanthemum plantlets acclimatized under intermittent mist, and maranta plantlets sustained less leaf damage when acclimatized in a shaded greenhouse.

The irradiance levels used during stage III of this experiment were found to have no effect on subsequent shoot and root elongation and leaf development. It has been concluded that the 3 irradiance levels did not have a carryover effect on carnation, chrysanthemum, or spathiphyllum because the levels were too low, and no carryover effect was observed on maranta possibly because insufficient time was allowed for differences to develop.
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