AN ABSTRACT OF THE THESIS OF

Kim E. Brainerd for the degree of Doctor of Philosophy in Horticulture presented on 3 December 1981

Title: Acclimatization of in vitro apple and plum trees to low relative humidity

Abstract approved

Leslie H. Fuchigami, Professor of Horticulture

Acclimatization of in vitro apples and plums was examined in four studies: 1) Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments, 2) Acclimatization of aseptically cultured 'Mac 9' apples to low relative humidity, 3) Stomatal functioning of aseptically cultured 'Mac 9' apple leaves in darkness, mannitol, ABA, and CO₂, and 4) Stomatal anatomy and the duration of acclimatization of in vitro 'Mac 9' apples. Leaf anatomy was examined using light microscopy. Acclimatization was observed by opening culture jar lids and measuring relative water content, percent of closed stomata in drying studies, darkness studies, and percent survival after a transplant study. Stomatal responses to darkness, mannitol, ABA, and CO₂ were evaluated by the percent of closed stomata. Stomatal anatomy was examined in light and scanning electron microscopy. Leaf anatomy of in vitro plants was significantly different from that of greenhouse plants. In vitro leaves had a single layer of ovoid shaped, small, openly spaced palisade cells. Greenhouse leaves had one to two layers of oblong, significantly longer, closely packed palisade cells. The % mesophyll air space was significantly greater in in vitro than greenhouse leaves. In vitro plants had less epicuticular wax than did greenhouse
leaves. The stomatal anatomy, however, of in vitro and greenhouse leaves was similar. No structural differences, which could prevent closure of in vitro stomata, were observed. Less than 10% of in vitro stomata closed after drying, darkness, mannitol, ABA, or CO$_2$ treatment; from 40 to 98% of treated greenhouse stomata closed. Lack of in vitro leaf stomatal closure caused significant water loss when plants were transferred to low RH. In vitro leaves lost 50% water, a critical injury level, in 30 min; greenhouse leaves required 90 min. Humidity acclimatization involved development of a stomatal closure mechanism. In vitro plants were acclimatized to low RH within 1 week in jars with lids removed. Open jars of in vitro plants acclimatized after 4 to 5 days exposure to 45% RH or after 2 days exposure to 65% RH.
ACCLIMATIZATION OF IN VITRO APPLE AND PLUM TREES
TO LOW RELATIVE HUMIDITY

by

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I dedicate this thesis to Rich, for his constant faith in me; and to the dreams which have changed my life.

Keb

Corvallis, Oregon
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature Review</td>
<td>2</td>
</tr>
<tr>
<td>I. Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments</td>
<td>13</td>
</tr>
<tr>
<td>II. Acclimatization of aseptically cultured apple plants to low relative humidity</td>
<td>21</td>
</tr>
<tr>
<td>III. Stomatal functioning of aseptically cultured apple leaves in darkness, mannitol, ABA, and CO$_2$</td>
<td>33</td>
</tr>
<tr>
<td>IV. Stomatal anatomy and the duration of acclimatization of in vitro 'Mac 9' apples</td>
<td>46</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>54</td>
</tr>
<tr>
<td>Appendix</td>
<td>58</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure | Page
--- | ---
1. Model of stomatal control, after Raschke (37, 38). | 8
2. Leaf cross sections from (A) approximately the third node of an aseptically cultured, (B) greenhouse transferred, and (C) field-grown 'Pixy' plum. The bar = 100 μm. | 19
3. Percent moisture loss of excised aseptically cultured (AC) leaves and greenhouse (GH) plant leaf disks for each drying treatment. Each point represents the mean of 5 samples. The vertical bars represent S.D. | 20
4. Percent stomatal closure of excised leaves from culture jars of 'Mac 9' apples exposed to 30 to 40 % RH for 0, 4, 5 days and from the greenhouse (GH), plotted by drying time after excision. Each point is the mean of 3 samples. The vertical bars represent S.D. | 30
5. Micrographs of imprints from abaxial surfaces 15 minutes after leaf excision of aseptically cultured 'Mac 9' apples exposed to 30 to 40 % RH for (A) 0 days, (B) 4 days, (C) 5 days, and (D) from the greenhouse. | 31
6. Rate of water loss plotted by percent stomatal closure for excised aseptically cultured (AC) and greenhouse (GH) 'Mac 9' apple leaves. Parallel regression lines were calculated from the multiple regression equation, $Y = 1.99 - 0.0055X - 1.192Z$, $R = 0.908**$. | 32
7. Light micrographs of butyl acetate leaf imprints of greenhouse (A) and aseptically cultured (B) 'Mac 9' apple stomata taken at 0400 PST. The bar = 10 μm. | 41
8. Percent stomatal closure plotted by time in darkness for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves. Each point represents the mean of 5 samples. | 42
9. Percent stomatal closure plotted by time in solutions of 1 M mannitol or distilled water for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves. Each point represents the mean of 5 samples. The vertical bars represent S.D. | 43
Figure 10. Percent stomatal closure plotted with -Log M ABA for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves treated for 1 hr. Values for control were taken from untreated leaves. Each bar represents the mean of 5 samples.

11. Percent stomatal closure plotted with 1 hr of 0.12 % atmospheric CO₂ treatment for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves and with untreated controls directly from plants. Each bar represents the mean of 5 samples.

# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Epidermal and palisade cell length and percent leaf mesophyll air space of aseptically cultured, greenhouse, and field-grown 'Pixy' plums.</td>
<td>18</td>
</tr>
<tr>
<td>2. Mean relative water content (RWC) for leaves 1 hr after excision from aseptically cultured or greenhouse 'Mac 9' apples exposed to 30 to 40 % RH for a given time.</td>
<td>29</td>
</tr>
<tr>
<td>3. Percent of closed stomata after 30 min of drying or darkness, percent of transplant survival, and plant height 6 weeks after transplanting for in vitro 'Mac 9' apples with jar lids removed for 0, 2, 4, or 6 days.</td>
<td>52</td>
</tr>
</tbody>
</table>
APPENDIX TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Analyses of variance for upper and lower epidermal cells and palisade parenchyma for aseptically cultured, greenhouse transferred, and field-grown 'Pixy' plums. LSD calculated at 1% level.</td>
<td>58</td>
</tr>
<tr>
<td>II. Friedman's test (28) and a multiple comparison technique based on Friedman's ranked sums (18) for percent air space of aseptically cultured, greenhouse, and field grown 'Pixy' plum.</td>
<td>59</td>
</tr>
<tr>
<td>III. Analysis of variance of the relative water content (RWC) for leaves 1 hr after excision from aseptically cultured 'Mac 9' apples exposed to 30 to 40% RH for 0, 6, 12, 24, 48, or 96 hr and from the greenhouse.</td>
<td>60</td>
</tr>
<tr>
<td>IV. Analysis of variance for a multiple regression equation to test the significance of percent stomatal closure ($%$SC) or constant water loss (CWL) after the effect of the other variable has been removed (44).</td>
<td>61</td>
</tr>
<tr>
<td>V. Analyses of variance for percent of closed stomata after 30 min drying, 30 min of darkness, and plant height 6 weeks after transplanting for in vitro 'Mac 9' apples with jar lids removed for 0, 2, 4, or 6 days.</td>
<td>62</td>
</tr>
<tr>
<td>VI. Percent of closed stomata for excised in vitro and greenhouse 'Mac 9' apple leaves treated for 24 hr in distilled water, $10^{-6}$ M nonactin, and $10^{-7}$ M nonactin.</td>
<td>63</td>
</tr>
<tr>
<td>VII. Percent of closed stomata for excised in vitro and greenhouse 'Mac 9' apple leaves treated for 12 hr in a closed etherized atmosphere and untreated (stomatal aperture counted upon excision from plant).</td>
<td>64</td>
</tr>
<tr>
<td>VIII. Percent of closed stomata for excised in vitro and greenhouse 'Mac 9' apple leaves frozen at $-40^\circ$C for 18 hr and thawed at $24^\circ$C for 6 hr prior to making leaf imprints.</td>
<td>65</td>
</tr>
</tbody>
</table>
PREFACE


The species name for apple is controversial. Some taxonomists refer to the apple as Malus domestica (Borkh.); others as Malus pumila (Mill); others as Malus sylvestris (Rydb.). This thesis uses Malus domestica (Borkh.).

The name for the clonal rootstock originally called Mac 9 (developed from an open pollination of cv. M9) was recently changed to cv. Mark. Two of the papers in this thesis were published before the name change. This thesis refers to clonal rootstock Mark as cv. Mac 9.
ACCLIMATIZATION OF IN VITRO APPLE AND PLUM TREES TO LOW RELATIVE HUMIDITY

INTRODUCTION

Procedures for in vitro culture of woody plants are becoming as plentiful as those for herbaceous plants. Success in the culture tube, however, does not assure vigorous plant growth in the greenhouse or field. A micropropagated shoot, with or without roots, must become adjusted, i.e., "acclimatized," to the greenhouse before becoming a saleable plant. This acclimatization includes 1) conversion to a completely autotrophic state (obtaining all organic metabolites through photosynthesis), 2) adaptation to higher light intensity, and 3) adjustment to air movement and gas exchange.

Adjustments to gas exchange, particularly water vapor loss, is paramount. In vitro plants grow in containers at nearly 100% relative humidity (RH) whereas daytime greenhouse or field environments usually range from 30 to 60% RH. Researchers and growers have successfully acclimatized in vitro woody plants by placing them in a humid chamber or on a mist bench for 2 weeks prior to transfer to a lower RH. Commercial producers have lost and continue to lose many plants due to water stress during acclimatization.

This research focused on the causes of water stress and the type and duration of environmental conditions needed for acclimatization.
Tissue culture, or in vitro plant propagation from undifferentiated callus cells, provides a rapid clonal multiplication technique for herbaceous plants (31). Woody plant propagation from callus is possible, but the in vitro culture of differentiated tissues, such as a nodal stem section, is more efficient (8). Commercial procedures for in vitro plant culture involves several sequential stages: Stage I - establishment of the propagule on sterile medium; Stage II - multiplication of propagula (shoot proliferation); Stage III - preparation for reestablishment of the plant in soil.

Stage III, according to Murashige (31), includes not only root production, but conversion of plants from the heterotrophic to the autotrophic state, and hardening of plants to withstand water stress and higher radiation levels. Natural plant adaptation to a changed environment is termed "acclimation;" plant adaptation where man intervenes, such as transferring aseptically cultured plants to a greenhouse, is termed "acclimatization" (Conover, 1977, at the Environmental conditioning symposium, Chicago, IL).

During the acclimatization of in vitro plants to low relative humidity (RH), plants may wilt rapidly, desiccate, and die (46). Only 59% of carnations (10) and 60% of thornless blackberries (7) survived when transferred to the greenhouse. If the water deficit is minimized by maintaining high humidity for 1 to 2 weeks after transfer, much higher percentages of survival were achieved (22, 39). Maintaining high humidity on a commercially large scale may be difficult, and acclimatization within culture jars would be preferable.
Several articles report less epicuticular wax formation on \textit{in vitro} plants and attribute wilting during transfer to peristomatal water loss (2, 45). Sutter and Langhans (45) and Fuchigami et al. (16) also mention that stomatal water loss should be investigated.

This review discusses environmental effects on leaf anatomy; normal mesophytic plant stomatal anatomy, development, and function; and indices of humidity acclimatization as background information for the articles presented in this thesis.

\textbf{Environmental effects on leaf anatomy.}

Plants of a single species grown in different environments, such as different soil moisture regimes, light intensities, or humidities, develop corresponding differences in leaf anatomy (13). \textit{In vitro} and greenhouse environments vary particularly in atmospheric humidity and light intensity. \textit{In vitro} plants are exposed to higher RH and lower light intensity than are greenhouse plants (45).

Available moisture influences leaf anatomy (13). Esau classified leaves or plants as xeromorphic if adapted to dry habitats, as mesomorphic if adapted to abundant soil moisture, or as hydromorphic if adapted to partial or complete submersion in water (13). Xeromorphic characteristics include a high ratio of leaf volume to surface, i.e., small thick leaves; layered palisade cells; small intercellular space volume; high stomatal frequency; abundant trichomes; thick cell walls and cuticles, when compared to mesomorphic leaves. \textit{Olea europaea} L., a xerophyte, had a frequency of 625 abaxial stomata per mm$^2$ (35). Esau describes mesophytic leaf anatomy (13). Some mesophytic plants, such as \textit{Prunus} and \textit{Malus}, have hypostomatic leaves,
i.e., have abaxial stomata only. Popham (35) reports 253 stomata per \( \text{mm}^2 \) for *Prunus domestica* and 246 for *Pyrus malus* L. (*Malus domestica* (Borkh.).) Hydromorphic leaves have stomata only on adaxial surfaces, and thick cuticles on both epiderms (42). Stomatal frequency of *Nymphaea alba* ranged from 150 to 460 stomata per \( \text{mm}^2 \) (42). The rate of stomatal to cuticular transpiration in *Nuphar lutea* varied from 10:1 in wind to 3:1 in still air (42).

Light also influences leaf anatomy (13). Leaves which develop in high light intensity are more xeromorphic than leaves in lower light intensity. Sun leaves have smaller, thicker leaves with more differentiated palisade tissues than do shade leaves of the same plant (13, 34). Plants adapted to high light intensity have many chloroplasts, and vertical orientation of chloroplasts and grana (9). Shade leaves have fewer chloroplasts and a horizontal orientation of chloroplasts and grana (9). Chloroplast or grana reorientation requires four to eight weeks, although other leaf anatomy characteristics, such as thick cell walls, do not change, and old sun leaves are replaced by new shade leaves prior to complete plant acclimation (9). Tarula et al. (48) observed that high light intensity increased stomatal length, width, and frequency in *Vigna unguiculata* L., although there was a significant interaction between light intensity and cultivar on stomatal frequency.

Whitecross and Armstrong (52) observed that under low light intensity leaves of *Brassica napus* L. seedlings developed fewer epicuticular wax rodlets and a simpler wax branching system. Pallardy and Kozlowski (33) concluded that low light intensity and high vapor
pressure deficit produced greater stomatal closure in *Populus* than was obtained under low light or high vapor pressure deficit alone. Baker (2) summarized environmental effects on leaf wax development by saying "an increase in radiant energy, a decrease in humidity, or a decrease in temperature induces the largest deposits of wax."

Eberhardt (11), Maximov (27), and Slavic (43) examined the effects of humidity on leaf anatomy. Maximov (27) generalized that "damp air had much the same effect as shade on the form and structure of plants." Plants grown in bell jars with high humidity had larger, thinner leaves, less cell differentiation, lower stomatal frequency, and thinner cuticles than did plants grown in dry air (11, 27). Slavic (43) observed corn growing in humid and dry air. He determined that transpiration rates for humid-air plants were 50% higher than dry-air plants when both were measured in dry air.

Several researchers, Sutter and Langhans (46), Grout and Aston (17), and Fuchigami et al. (16), examined the epicuticular wax formation of plants regenerated in *vitro*. In these studies, scanning electron micrographs showed no structured epicuticular wax on in *vitro* plants, although increased amounts of structured wax occurred 2.5 to 3 weeks after transplanting to a greenhouse (17, 46). Grout and Aston (17) observed that stomata on both leaf surfaces closed within 30 minutes after removal from the culture jar, and concluded that the water loss of newly transferred in *vitro* plants resulted from poorly developed epicuticular wax. Sutter and Langhans (46) did not examine stomatal functioning, but stated, "the low survival rate of carnation plantlets regenerated in *vitro* may be explained by
the lack of epicuticular wax structure which results in excess desiccation when they are transferred from *in vitro* to the greenhouse."

Fuchigami et al. (16) examined water loss weight changes in excised non-acclimatized leaves from *in vitro* plums. The leaves were coated with silicon rubber on adaxial, abaxial, and both surfaces. They determined that water loss occurred primarily from the abaxial surface, and concluded that water loss was due either to abaxial cuticular or stomatal transpiration (16).

**Stomatal anatomy, development, and functioning.**

This section presents definitions, observations, and theories of stomatal anatomy, development, and the normal closure mechanism in mesophytic plants. These theories on closure contrast greatly with the observations of *in vitro* stomata as reported in the second and third papers of this thesis.

A "stoma" consists of a pair of guard cells (GC) and a common pore (13, 15). "Neighboring cells" are adjacent to the guard cells but are not structurally different from other epidermal cells. "Subsidiary cells" are adjacent to the GC but are structurally different from other epidermal cells. The GC are formed from a single division of the guard mother cell (GMC) (13). Prior to this division a cell plate is formed and microtubules are oriented parallel to the cell plate (32). Cellulose microfibrils are deposited parallel to the microtubules, thickening the ventral GC walls (walls towards the pore) (32). A radial orientation of other microfibrils allows elastic stretching of the GC walls (38). Potassium ions accumulate inside the GMC, and the subsequent increase in turgor pressure produces the
initial opening of the stomatal pore (32). Guard cells are nucleated, vacuolated, contain many mitochondria, some chloroplasts, prominent plastid starch grains, and cytoplasm (1).

Stomatal development occurs before completion of the leaf meristematic activity (13). The most developed stomata are at the leaf apex and development progresses toward the leaf base (13). In dicot leaves, different developmental stages are present within a given area of leaf. Sachs (40) concluded that stomatal patterns in Sedum were a result of cell lineage. Few, if any, new stomatal complexes developed while the leaf was "still in the bud" (42).

Zelitch (54), Meidner and Mansfield (30), Raschke (37), Allaway and Milthorpe (1), and Raschke (38) wrote reviews on stomatal functioning. Raschke (37) developed a model (Fig. 1) for stomatal functioning, which he refined in 1979 (38). In the model guard cell turgor mechanically determines stomatal aperture (37). High cell turgor produces large apertures; low GC turgor produces stomatal closure. Stafelt (45) concluded that GC turgor could be regulated directly by a hydropassive water flow from GC to subsidiary cells, or hydroactively, by movement of ions into or out of the GC, effecting a hydrostatic response. Potassium ion accumulation in open GC has been observed in more than 50 species (37). Malate also accumulated in open GC, supplying about 40 to 50% of the counter charges for K⁺; other organic anions and Cl⁻ also acted as counter anions (37).

The primary purpose of stomatal operation is gas exchange (38). Most recent theories (14, 38) conclude that stomatal aperture minimizes H₂O loss while maximizing CO₂ uptake. This optimization func-
Figure 1. Model of stomatal control after Raschke (37, 38).
tion produces a constant gain ratio, $\frac{dE}{dA}$, where $E$ (evaporation) is the molar flux of water and $A$ (assimilation) is the molar flux of CO$_2$ (38). Farquar et al. (14) measured a constant gain ratio in Nicotiana glauca L. and Corylus avellana L. after changing the relative humidity.

Stomata close in response to several conditions within and external to the plant (38). Stomata respond differently to a single stimulus, such as light, if other effects, such as water stress, are uncontrolled (38). Also, the conflict between opening the stomata to maximize CO$_2$ uptake and closing the stomata to minimize H$_2$O loss prevents a long term steady state stomatal condition (38).

Stomata tend to close under several physiological and environmental conditions, including diurnal fluctuations, darkness, high CO$_2$ concentration, high endogenous or exogenous abscisic acid, water stress, or cell death. Stomata tend to open during the day and close during the night (54). This diurnal fluctuation is a combination of indirect responses to light-dark, high sub-stomatal CO$_2$ concentration, temperature, water stress, and internal rhythms (29).

Stomatal response to light or darkness is indirect, mediated by sub-stomatal CO$_2$ concentration (37). During light, CO$_2$ is low due to photosynthetic fixation. During darkness, sub-stomatal CO$_2$ increases because CO$_2$ fixation stops and respiratory CO$_2$ increases. The increase in CO$_2$ concentration is sensed in the GC (37, 38) possibly by pH effects on enzymes, e.g., carboxylase, preventing GC K$^+$ accumulation and GC turgor. Zelitch (54) observed that stomata of tobacco leaf disks placed in the dark closed within 30 minutes.
High atmospheric CO₂ concentration increases sub-stomatal CO₂ concentration, and the GC CO₂ sensor system closes the stomata (38). In plants well supplied with water, however, completely closed stomata may not occur in darkness or in high atmospheric CO₂ (37).

High abscisic acid (ABA) concentration produces stomatal closure by altering GC membrane permeability (50). H⁺/K⁺ exchange between GC and neighboring cells is inhibited and malate leakage from GC is promoted by ABA (50). Wilty tomato mutants contain low endogenous ABA and their tendency to wilt under water stress is reduced by exogenous ABA application (47). New ABA is formed within 7 minutes in wilted leaves whose total water potential drop below -10 atm (-0.98 MPa) (37).

Tissue water stresses, such as low water availability to roots, excision and desiccation of leaves, high vapor pressure deficits between attached leaf and air, or solutions which have negative osmotic potentials in which leaves or epidermal strips are placed, cause stomatal closure. Transpiration rates of soybeans decrease from 8 × 10⁻³ to 1 × 10⁻³ cm³ plant⁻¹ sec⁻¹, as the total soil water potential drops from -2 bars (-0.2 MPa) to -14 bars (-1.4 MPa) (3).

Losch and Schenk (25) observed that stomata in epidermal strips of Valerianella locusta L. closed within 20 to 30 minutes after the atmospheric water vapor deficit was increased by 5 or 7 mg H₂O dm⁻³ air. They also observed that K⁺ content of GC, as measured by the Macallum staining method, decreased 20 minutes after stomatal closure due to the decreased humidity. They concluded that K⁺ flux out of the GC was not correlated to stomatal closure under decreased humid-
ity. Potassium flux was, however, correlated to closure in decreased light (25).

Zelitch (54) observed that soaking tobacco leaf disks in osmotically active substances at high concentrations, such as 0.7 M sucrose, completely prevented stomatal opening.

Raschke (37) concluded that continual stomatal oscillations were advantageous to plants in a continuously changing environment. Stomatal regulation by many feedback loops from multiple environmental stimuli maintained favorable ratios of transpiration to \( \text{CO}_2 \) assimilation (37). The third paper of this thesis examines the application of these stomatal closure theories to plants grown in vitro.

Indices of humidity acclimatization

Several quantitative methods of measuring injury of in vitro woody plants removed from the culture jar were developed by Kobayashi et al. (24). They used ethylene/ethane emission and electrolytic conductivity to detect cell injury and death during water stress of excised aseptically cultured plum leaves. Ethane production and electrolytic conductivity were highly correlated and indicated an increase in cell injury and death between 50 to 72% leaf water loss (24).

Edwards and Meidner (12) examined the effect of ambient humidity by measuring relative water content (RWC) and leaf water potentials of *Tradescantia virginiana* L., *Saxifraga tomentosa* L., and *Polypodium vulgare* L. They grew the plants in low relative humidity, changed the RH, and examined the effect on RWC and leaf and epidermal water potentials. They observed that the RWC and leaf water potentials did
not correlate with stomatal response to water stress and that the GC water potential, which is difficult to measure, determined the humidity response (12).

Many methods are used to determine stomatal closure. Epidermal strips from plants such as *Tradescantia*, *Valerianna*, *Zea*, and others whose epidermis can readily be removed, are used to examine stomatal response (38). Sampson (41) and Zelitch (54) discuss the technique of making leaf surface impressions in silicon rubber (negatives) and examining cellulose acetate replicas (positives) under the light microscope, to measure stomatal aperture. Wilson (53) mounted epidermal strips of *Commelina communis* L. on a microscope slide and directly measured stomatal aperture. Slavic (43) measured leaf diffusive resistance to determine stomatal response to humidity changes. Silicon-rubber and butyl acetate leaf imprints were used to measure stomatal aperture in the present study.

In summary, many articles have examined *in vitro* propagation methods, environmental effects on plant anatomy, and plant response to water stress. This thesis combines these three areas to examine the acclimatization of *in vitro* plants to low relative humidity.
I. Leaf Anatomy and Water Stress of Aseptically Cultured 'Pixy' Plum Grown under Different Environments

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Additional index words. Prunus insititia

Abstract. This study examined the leaf anatomy and water stress of Prunus insititia L. cv. Pixy grown in aseptic culture before and after transfer to the greenhouse and grown in a layerage bed in the field. The depth of palisade cells was significantly less in aseptically cultured plantlets than in greenhouse transferred plants, and less in greenhouse transferred than in field-grown plants. Percent mesophyll air space was greater in plantlet than in plant leaves. Upper or lower leaf epidermal cell length of plantlets or field-grown plants was not significantly different. Stomatal frequency for plantlet leaves was significantly less (about 150 stomata per mm²) than that of plant leaves (300 stomata per mm²). Excised plantlet leaves lost greater than 50% of total leaf water content within 30 min; excised greenhouse leaves lost 50% after 90 min.

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2Graduate Student, Associate Professor, and Lab Technicians.
3Appreciation is extended to Microplant Nursery in Gervais, OR, and to Dr. T.Y. Cheng and Dr. M.M. Thompson for their assistance.
Plantlets transferred from aseptic culture to the greenhouse become water stressed and continued growth or survival may decrease (46). The purpose of this study was to contrast the leaf anatomy and water stress of 'Pixy' plum plants grown in aseptic culture before and after transfer to the greenhouse with those propagated from a layerage bed in the field.

Leaf anatomy is influenced by light and moisture (13). Leaves developing at high light intensities have more and larger palisade cells than those in shade (13). Leaves developing under low soil moisture have smaller intercellular spaces and higher stomatal frequencies than those grown with ample moisture (13, 26, 49). Plants grown at high relative humidity have poorly developed epicuticular waxes (46) and higher transpiration rates in dry air than do dry-air plants (43).

In the summer of 1979, 'Pixy' plum plants were aseptically cultured by Cheng's (8) method. One half of the rooted plants were transferred to 1 peat: 1 perlite (v/v), fertilized weekly with 473 ppm N, 456 ppm P, and 868 ppm K, and grown in a greenhouse at 27°C and normal day length for 3 weeks. Field plants were grown in layerage beds at Oregon Rootstocks, Inc., in Gervais, Oregon.

Leaves were collected from the third to the fifth node of aseptically cultured plants. Leaves initiated and matured in the greenhouse were collected from transferred plants. Fully expanded leaves were collected from field grown plants. Samples were fixed in formalin-aceto-alcohol, embedded in paraffin, sectioned, and stained in safranin and fast-green (21), and observed with a light microscope.
Palisade, upper epidermal, and lower epidermal cell lengths were measured on 25 cells of each plant leaf. The percent mesophyll air space for each leaf was calculated using an ocular grid. Ten fields of mesophyll cells were examined in 10 sections of each leaf and plant type. To obtain the percent air space, the number of squares (25 X 25 μm) of extracellular space for each field was counted, divided by the total number of squares, and multiplied by 100. The percent air space was analysed for treatment differences using Friedman's test (28) and a multiple comparison based on Friedman's rank sums (18). Stomatal frequency was determined by microscopic examination of silicon-rubber, butyl-acetate leaf imprints. Stomatal frequency (the number of stomata per mm$^2$ of leaf surface) was calculated for 5 randomly chosen microscope fields on each of 20 different plantlet and greenhouse transferred 'Pixy' leaves, and was analysed using a t-test.

For the water stress study, 45 leaves about 20 X 7 mm were excised from 'Pixy' plantlets and 45 leaf disks 15 mm in diameter were cut from leaves initiated and grown in the greenhouse on transferred plants. Fresh weight of each was determined and samples were placed abaxial side up in an open petri dish. Five samples from each plant type were dried at 21°C for 15, 30, 45, 60, 75, 90, 105, and 120 min and weighed. Oven dry weight was obtained after 48 hr of sample drying at 60°C. The percent moisture loss for each sample was calculated by dividing the leaf moisture content after drying at 21°C by the original moisture content, and multiplying by 100.

The length of palisade cells was significantly less in plantlets
then in leaves of transferred plants (Table 1). Leaf palisade cells from transferred plants were significantly shorter than those of field-grown plants (Table 1). However, the upper epidermal cell length of transferred plants was significantly less than those of the other two plant types (Table 1). Percent air space in mesophyll tissue was significantly greater in plantlets than in transferred plants (Table 1, Fig. 2A, 2B). Mesophyll air space of transferred plants was not significantly different from that of fully expanded field-grown leaves (Table 1, Fig. 2B, 2C). Examination of the most apical leaves indicated that field grown plants had significantly less mesophyll air space than the other plant leaves (data not shown). Stomatal frequency was significantly less for plantlet (150 ± 60 stomata per mm$^2$) than for transferred leaves (300 ± 60 stomata per mm$^2$).

Excised plantlet leaves lost water more rapidly than transferred plant leaves (Fig. 3). Plantlet leaves lost more than 50% leaf moisture 30 min after excision; greenhouse transferred plant leaf disks lost about 50% moisture after 90 min. Analyses by ethylene/ethane emission and electrolyte leakage indicated that excised 'Pixy' plum leaves were injured at water stress of 50% moisture loss or greater (24). A 50% moisture loss occurred about 1 hr sooner in excised plantlet leaves than in transferred plant leaf disks. We conclude that excised plantlet leaf cells were injured about 1 hr sooner than transferred leaf disks.

Our leaf anatomy data agree with those of similar studies (13, 26, 43, 49). These results indicate that plantlets with smaller palisade cells, larger intercellular spaces, and lower stomatal frequency were injured by water stress more rapidly than transferred
plants which were acclimatized to the greenhouse. Further studies will focus on stomatal and cuticular water loss in relation to plantlet transpiration before, during, and after transfer to the greenhouse.
Table 1. Epidermal and palisade cell length and percent leaf mesophyll air space of aseptically cultured, greenhouse, and field grown 'Pixy' plums.

<table>
<thead>
<tr>
<th>Source of plant</th>
<th>Upper epidermis</th>
<th>Lower epidermis</th>
<th>Palisade parenchyma</th>
<th>Air space (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aseptically cultured</td>
<td>26.4a&lt;sup&gt;z&lt;/sup&gt;</td>
<td>13.8a&lt;sup&gt;z&lt;/sup&gt;</td>
<td>20.2a&lt;sup&gt;z&lt;/sup&gt;</td>
<td>20.6a&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>19.7b</td>
<td>15.8a</td>
<td>31.8b</td>
<td>13.3b</td>
</tr>
<tr>
<td>Field grown</td>
<td>26.2a</td>
<td>15.8a</td>
<td>76.9c</td>
<td>9.5b</td>
</tr>
</tbody>
</table>

<sup>z</sup>Mean separation in the columns by LSD, 1% level. Each mean was calculated from 25 replicates.

<sup>y</sup>Mean separation in the column by a multiple comparison technique based on Friedman's rank sums (18,28), 1% level. Each mean was calculated from 10 replicates.
Figure 2. Leaf cross sections from (A) approximately the third node of an aseptically cultured, (B) greenhouse transferred, and (C) field-grown 'Pixy' plum. The bar = 100 μm.
Figure 3. Percent moisture loss of excised aseptically cultured (AC) leaves and greenhouse (GH) plant leaf disks for each drying treatment. Each point represents the mean of 5 samples. The vertical bars represent S.D.
II. Acclimatization of Aseptically Cultured Apple Plants to Low Relative Humidity

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Additional index words. Malus domestica, water stress, relative water content, tissue culture

Abstract. Aseptically cultured Malus domestica (Borkh.) 'Mac 9' plants were exposed to 30 to 40 % relative humidity (RH) for 0 to 6 days. The relative water content (RWC) and percent stomatal closure were measured on leaves excised from plants exposed to low humidity and from greenhouse acclimatized controls. Both RWC and percent stomatal closure successfully monitored acclimatization. The RWC of excised leaves exposed to low RH for 0 to 1 day was significantly higher than that of leaves exposed from 4.5 days or greenhouse acclimatized plants. Speed of stomatal closure upon leaf excision increased with the duration of plant exposure to low humidity. The rate of water loss from excised apple leaves was linearly related to the stomatal closure. Aseptically cultured plant (ACP) leaves consistently lost more water than greenhouse leaves at corresponding

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2Graduate student and Associate Professor. The authors thank Microplant Nursery of Gervais, Oregon, and the Oregon Association of Nurserymen for providing assistance.
percentages of stomatal closure. These results indicated that ACP leaves can be acclimatized to low humidity within 4 to 5 days of exposure to 30 to 40 % RH and that low humidity acclimatization involved development of an accelerated stomatal response.

Introduction

Both acclimation and acclimatization are terms which describe the process of adaptation of an organism to an environmental change. Acclimation is a process regulated by nature; acclimatization is a process regulated by man. These definitions were accepted by the Environmental Conditioning Symposium of 1977 in Chicago, Illinois. In this study, humidity acclimatization refers to the process by which plants grown in high relative humidity (RH) adapt to a low RH. Few methods exist for assessing humidity acclimatization.

Aseptically cultured plants (ACP) grown in high RH often sustain severe water stress when exposed to lower RH. This water stress is a major cause (5, 46) of transplanting shock, e.g., dieback or death of ACP when transferred to the greenhouse.

ACP leaves differ anatomically from those of greenhouse plants (5). ACP leaves may have less epicuticular wax (17, 46), smaller palisade cells (5), and more mesophyll air space (5). The water stress of high RH plants when transferred to a lower RH has been attributed to poorly developed epicuticular and cuticular waxes (2, 5, 46). Sutter and Langhans (46) and Brainerd et al. (5) have suggested, however, that slow stomatal response may also contribute to water stress in ACP removed from the culture environment. A preliminary study indicated that excised ACP leaves were significantly
more stressed, i.e., lost more water and had more open stomata after 1 hr of holding at 50 % RH, than were excised greenhouse leaves (5).

The objectives of this study were to develop methods for measuring humidity acclimatization of ACP and to determine if stomatal functioning is involved in acclimatization to low humidity.

Materials and Methods

Relative water content (RWC) and stomatal closure were evaluated as indices of acclimatization. Leaves were held for up to 1 hr at approximately 40 % RH prior to calculating RWC or stomatal closure.

The RWC measurements were made between June 21 and 27, 1980. Rooted 'Mac 9' ACP for this study were grown in 8.5 x 9.0 cm culture jars under a 16 hr photoperiod of cool white florescent light at an intensity of 150 μE m⁻² s⁻¹, at 26°C. The RH in the culture jars was near 100 %. The RH in the growth room ranged from 30 to 40 % as measured by a Beckman Humi-Check II Hygrometer (Beckman Co., Cedar Grove, N.J.). Rooted ACP transferred from culture jars to pots containing peat:perlite (v/v) 1 month earlier were grown under natural photoperiod and light intensity in the greenhouse at approximately 50 % RH, and were considered "acclimatized."

To avoid root damage during transplanting, ACP were left in the agar and the culture jar lids were removed for either 0, 6, 12, 24, 48, or 96 hrs in the 30 to 40 % RH growth room. Ten ml of distilled water was added daily to the open jars to prevent drying of the media.

Five leaf disks (replicates), 1 cm in diameter, were removed from plants at the selected experimental times, were placed abaxial side up on a Petri dish in the lab, held for as long as 1 hr at about
40 % RH, and allowed to transpire. Disks were weighed, and soaked in distilled water for 4 hr. Preliminary experiments determined that 1 hr of holding and 4 hr of soaking distinguished acclimatized from non-acclimatized leaves. Disks were weighed after soaking to determine saturated weight, dried in an oven at 60°C for 48 hr, and re-weighed. Relative water content was calculated as:

\[
RWC = \frac{(Saturated \ Weight - Weight \ After \ Holding)}{(Saturated \ Weight - Oven \ Dry \ Weight)} \times 100
\]

Analysis of variance and LSD separated mean differences at 1 % level.

In September 1980 a similar open jar study was conducted to test stomatal closure as an index of acclimatization. Plants were identically cultured except that greenhouse "acclimatized" control plants had been grown for 2.5 months. Culture jar lids were removed for 0, 1, 2, 3, 4, 5, or 6 days. Three jars (replicates), each containing 10 plants, were randomly allocated to each treatment.

After the open jar treatment, stomatal closure was observed by using leaf impressions. The five largest leaves from the experimental plants were removed and placed abaxial side up in Petri dishes at approximately 40 % RH for 0, 15, 30, 45, or 60 min. Silicon-rubber and butyl acetate leaf impressions were made after each time period, mounted on slides, and stomatal closure was determined similarly to Zelitch's method (54). Stomata with apertures less than 2 μm were considered closed (54). The number of closed stomata from the first 100 observed in the middle of the leaf within 0.5 mm of the main vein was expressed as percent stomatal closure for that leaf.
Percent water loss within 1 hr was determined for each excised leaf by collecting and weighing the five largest leaves from each of four experimental plants. One leaf from each jar was weighed and dried abaxial side up for 0, 15, 30, 45, or 60 min at 50 % RH. The leaves were weighed after drying, were oven dried at 60°C for 48 hr, and reweighed. The percent water loss during the holding treatment was calculated as:

\[
\text{Percent Water Loss} = \frac{(\text{Fresh Weight} - \text{Weight After Holding})}{(\text{Fresh Weight} - \text{Oven Dry Weight})} \times 100
\]

The percent water loss for each leaf was divided by the drying time to obtain the rate of water loss.

A multiple regression was computed to determine if the rate of water loss was affected by percent stomatal closure or plant type (aseptically cultured or greenhouse). For plant type, tissue culture plants were assigned the value 0, and greenhouse plants assigned value 1. F-tests were used to check for significance of slope and constant difference of the regression line.

**Results and Discussion**

The RWC of excised ACP leaves in jars open from 0 to 24 hr were significantly higher (LSD, 1 %) than those in open jars for 96 hr and from the greenhouse controls (Table 2).

The stomatal response after 5 and 6 days of open jar treatment was similar to the greenhouse control but differed significantly from the stomatal response of days 1, 2, 3, and 4. The stomatal response of the latter four treatments were similar (Fig. 4). Data for 1, 2,
3, and 6 days were excluded from Fig. 4 to avoid confusion.

Less than 20% of the stomata of leaves exposed from 0 to 4 day open jar treatment closed within the first 15 min (Fig. 5A, 5B) although 81% of the stomata of leaves exposed for 5 or 6 days or from the greenhouse closed in that time (Fig. 4, 5C, 5D).

The percent water loss of excised leaves from aseptically cultured and greenhouse control 'Mac 9' plants were similar to those reported on 'Pixy' plum (5). In plums, excised leaves from ACP lost more than 40% water within 15 min, while greenhouse leaves lost less than 10% in the same time. Electrolyte leakage and ethylene/ethane analyses (24), indicated that cells were injured at 50% water loss, which occurred from 15 to 30 min after excision of ACP leaves, but did not occur within 60 min in greenhouse acclimatized controls.

The rate of water loss for excised ACP leaves was much higher while percent stomatal closure was much lower, than that of greenhouse plant leaves (Fig. 6). A multiple regression equation of: $Y = 1.99 - 0.0055 X - 1.19 Z$ with $R = 0.908$ was calculated where $X$ represented percent stomatal closure and $Z$ represented plant type (ACP = 0; greenhouse plant = 1). Substituting for $Z$, the ACP regression line was: $Y = 1.99 - 0.0055 X$; the greenhouse regression line was: $Y = 0.80 - 0.0055 X$ (Fig. 6).

The slope was highly significant (F-test on individual variable effects, 1% level) indicating that the rate of water loss was linearly related to the percent stomatal closure in excised apple leaves.

The highly significant constant difference (F-test, 1% level)
indicated that at corresponding percentages of stomatal closure, ACP leaves consistently lost more water than did greenhouse leaves. This higher constant water loss may be due to larger cuticular loss, higher stomatal frequency, or larger stomatal apertures in ACP leaves than in greenhouse plant leaves.

Sutter and Langhans (46) determined that during 2.5 weeks in the greenhouse, structured epicuticular wax increased on ACP carnation leaves. Grout and Aston (17) observed that considerable wax was evident on both surfaces of *Brassica oleracea* leaves 10 days after transfer from aseptic culture to lower RH. These waxes developed as part of the acclimatization process. Wax development which occurred 10 or more days after transfer would not explain the difference in RWC or percent water loss between aseptically cultured 'Mac 9' apple plantlets and those after 4 or 5 days of exposure to 30 to 40% RH. Fuchigami et al. (16) observed that water loss of excised aseptically cultured 'Pixy' plum leaves occurred from the abaxial surface, where the stomata were located.

Grout and Aston (17) also mentioned that "the stomata on both leaf surfaces of all plants examined were closed at the time of first weighing ... approximately 5 min after exposure to experimental conditions." Grout and Aston (17) did not mention how stomata closure was measured or when the guard cell aperture was considered closed. None of the excised apple leaves that we observed, including those from the greenhouse acclimatized controls, had 100% closure (stomatal apertures less than 2 μm).
We observed that short-term humidity acclimatization involving a change in stomatal function occurred less than 5 days after exposure to low humidity. Other longer term acclimatization involving wax development (17, 46) and chloroplast reorientation (9) occurred after at least 10 to 14 days.

In conclusion, RWC and stomatal closure counts on excised leaves successfully monitored humidity acclimatization. The RWC technique after 15 min of holding (Fig. 4) was particularly useful in separating acclimatized from non-acclimatized plants. Stomatal closure counts enabled direct observation of treatment effects on stomata. Slow stomatal response to drought stress was a significant cause of water loss in ACP leaves. ACP leaves acclimatized within 4 to 5 days to 30 to 40 % RH. Short-term humidity acclimatization involved a change in stomatal function.
Table 2. Mean relative water content (RWC) for leaves 1 hr after excision from aseptically cultured or greenhouse 'Mac 9' apples exposed to 30 to 40% RH for a given time.

<table>
<thead>
<tr>
<th>Treatment Time (hr)</th>
<th>RWC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75.2 A</td>
</tr>
<tr>
<td>6</td>
<td>75.6 A</td>
</tr>
<tr>
<td>12</td>
<td>71.2 A,B</td>
</tr>
<tr>
<td>24</td>
<td>69.6 A,B</td>
</tr>
<tr>
<td>48</td>
<td>61.8 B,C</td>
</tr>
<tr>
<td>96</td>
<td>52.3 C</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>53.4 C</td>
</tr>
</tbody>
</table>

Each value is the mean of five samples. Means were separated by LSD, 1%.
Figure 4. Percent stomatal closure of excised leaves from culture jars of 'Mac 9' apples exposed to 30 to 40 % RH for 0, 4, 5 days and from the greenhouse (GH), plotted by drying time after excision. Each point is the mean of 3 samples. The vertical bars represent S.D.
Figure 5. Micrographs of imprints from abaxial surfaces 15 minutes after leaf excision of aseptically cultured 'Mac 9' apples exposed to 30 to 40 % RH for (A) 0 days, (B) 4 days, (C) 5 days, and (D) from the greenhouse.
Figure 6. Rate of water loss plotted by percent stomatal closure for excised aseptically cultured (AC) and greenhouse (GH) 'Mac 9' apple leaves. Parallel regression lines were calculated from the multiple regression equation, 
\[ Y = 1.99 - 0.0055X \]

\[ Y = 0.80 - 0.0055X \]

where \( Y \) is the rate of water loss (mg H\(_2\)O/mg dry wt/min) and \( X \) is the percent stomatal closure. The regression equations are: 
- AC: \( Y = 1.99 - 0.0055X \)
- GH: \( Y = 0.80 - 0.0055X \)

R = 0.908**.
III. Stomatal Functioning of Aseptically Cultured Apple Leaves in Darkness, Mannitol, ABA and CO₂

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Abstract. Aseptically and greenhouse cultured Malus domestica (Borkh.) 'Mac 9' leaves, in separate experiments, were excised and subjected to as much as 4 hr of darkness; 4 hr of 1 M mannitol; 1 hr of $10^{-4}$ to $10^{-7}$ M cis-trans abscisic acid (ABA); or 1 hr of 0.12 % CO₂. Stomatal closure was determined by microscopic examination of butyl-acetate leaf imprints. In all treatments, less than 5 % of the stomata from aseptically cultured leaves were closed. In contrast, an average of 96 % of the stomata from greenhouse acclimatized leaves closed after 4 hr of darkness; 56 % closed after 4 hr in a 1 M mannitol solution; 90 % closed after 1 hr in a $10^{-4}$ M ABA solution; and 61 % closed after 1 hr in 0.12 % CO₂. Stomata of aseptically cultured apple leaves did not have a closure mechanism, but acquired a closure mechanism during acclimatization. Lack of stomatal closure in aseptically cultured plants was the main cause of rapid water loss during transfer to low relative humidity. We believe that stomata in aseptic culture did not develop a closure mechanism because the usually constant ratio of CO₂ gain to water loss was not applicable in the closed culture jar environment.

INTRODUCTION

Survival of aseptically cultured plants in low humidity requires
acclimatization to prevent water loss. This acclimatization involves thickening of epicuticular wax (2, 16, 46) and changes in stomatal functioning (3, 4, 16). In preliminary studies we observed that stomata of aseptically cultured plum leaves, grown at 26°C, under 100% RH, and 100 μE m⁻² s⁻¹ of a 16 hr photoperiod fail to show the nocturnal closure pattern of greenhouse leaves (Fig. 7). We also observed that stomata of leaves excised from aseptically cultured plants did not close after a 2 hr exposure to 45% RH during which 95% of the leaf moisture was lost (5). The closure mechanism for stomata of aseptically cultured leaves may be different than that of acclimatized greenhouse and other mesophytic leaves.

Stomatal movement controls the rate of CO₂ uptake and the water loss by plants (38). Optimal stomatal aperture minimizes water loss and maximizes carbon gain, keeping the gain ratio of transpiration rate to assimilation rate (dE/dA) constant (14, 38). Recent hypotheses (38) suggest that plants operate stomata to maintain a constant dE/dA. Farquhar et al. (14) observed that Nicotiana glauca L. and Corylus avellana L. stomata in light saturated conditions tended to have a constant dE/dA as humidity was decreased.

We were interested to know if the constant gain ratio also occurred to leaves of in vitro plants. The objectives of this study were to contrast the response of stomata from in vitro and greenhouse plant leaves after treatments of darkness, mannitol, ABA, or high CO₂. MATERIALS AND METHODS

'Mac 9' apple plants, obtained from Microplant Nursery, in Gervais, Oregon, were used for all experiments. Aseptically cultured
plants were grown for about 2 months at 25°C under 16 hr photoperiod of fluorescent light of approximately 100 μE m⁻² s⁻¹, in 9 x 8.5 cm jars of root promoting media.

Greenhouse acclimatized plants were propagated similarly and grown for up to 9 months in peat:perlite (v/v), under natural photoperiod, between 18 and 24°C. Five culture jars, each containing 10 plants, and five greenhouse plants were used for each experiment. One leaf was excised from a plant in each jar or greenhouse grown plant so that each treatment had five replicates. Open or closed stomata were evaluated by microscopic examination of butyl acetate leaf imprints. These imprints were made from silicon-rubber (Dow-Corning compound 3110 RTV) imprints taken from treated leaves. The number of closed stomata, with apertures less than 2 μm (54), from the first 100 stomata counted, estimated the percent stomatal closure for that replicate.

**Experiment 1 - Stomatal responses to darkness**

At 1500 PST, on 1 April 1981, plants were placed in a photographic darkroom. After 0, 15, 30, 45, 60, 120, and 240 min of darkness, one leaf was removed from each culture jar and greenhouse plant, and silicon-rubber leaf imprints were made.

**Experiment 2 - Stomatal responses to mannitol**

On 10 May 1981, at 0900 PDT, Eight leaves were removed from each of five aseptically cultured and greenhouse plants. One leaf from each plant was placed abaxial side down for 0, 1, 2, 3, or 4 hr in a Petri dish containing distilled H₂O or 1 M mannitol, at 22°C under 150 μE m⁻² s⁻¹. Leaf imprints were taken before and after
the treatment.

Experiment 3 - Stomatal responses to ABA

On 17 April 1981, at 1100 PST, one leaf was removed from each of five aseptically cultured and greenhouse plants and placed abaxial side down in a Petri dish containing distilled H$_2$O, 10$^{-4}$ M ABA, 10$^{-5}$ M ABA, 10$^{-6}$ M ABA, or 10$^{-7}$ M ABA. Leaf imprints were taken before and after 1 hr of soaking at 22°C under 150 µE m$^{-2}$ s$^{-1}$.

Experiment 4 - Stomatal responses to CO$_2$

On 17 May 1981, at 1000 PDT, five aseptically cultured plants were removed from separate culture jars by cutting an agar cube surrounding the plant roots. The five plants in the agar cubes were placed in Petri dish covers in a 5 l glass chamber at nearly 100% RH. Five acclimatized greenhouse plants in 5 x 5 cm pots of peat:perlite were also placed in the chamber. One leaf was removed from each plant and imprints were made to determine stomatal aperture prior to the CO$_2$ treatment. The chamber was closed and a mixture of 0.12% CO$_2$, 22% O$_2$, and balance N$_2$, was pumped through the chamber at a rate of 6 l hr$^{-1}$ (1.6 ml s$^{-1}$), for 1 hr, under 150 µE m$^{-2}$ s$^{-1}$. Leaves were then removed from each plant and stomatal imprints were made.

RESULTS AND DISCUSSION

In this study, greenhouse acclimatized apple stomata closed in response to darkness, water stress caused by negative osmotic potential, exogenously applied ABA, or high atmospheric CO$_2$. In contrast, aseptically cultured apple leaf stomata remained open in all treatments. In greenhouse apple leaves, an average of 96% of the stomata
closed after 4 hr of darkness (Fig. 8); 56% closed after 4 hr in 1 M mannitol (Fig. 9); 90% closed after 1 hr in $10^{-4}$ M ABA (Fig. 10); 61% closed after 1 hr in 0.12% CO$_2$ (Fig. 11). Fewer than 5% of aseptically cultured stomata had apertures less than 2 $\mu$m in all treatments (Fig. 8, 9, 10, 11). Stomatal aperture of aseptically cultured plants was not reduced by a five min exposure to 5% CO$_2$ (data not shown).

Guard cell turgor physically controls stomatal aperture (37). In more than 50 species, stomatal closure involves a K$^+$ flux followed by a water flux out of the guard cell (37). Guard cell turgor decreases and stomatal aperture is reduced. Environment, such as darkness (54), water stress (5, 37), exogenous ABA (19, 20, 50), or high atmospheric CO$_2$ (35, 52) indirectly causes decreased guard cell turgor and stomatal closure.

During darkness, CO$_2$ fixation decreases, sub-stomatal CO$_2$ increases and a CO$_2$ sensor in the guard cell causes stomatal closure (37). Zelitch (54) observed that stomata of tobacco leaf disks floating on water closed completely after 30 min of darkness.

Water stress causes either hydropassive, water movement without ion movement, or hydroactive, water movement involving transport of ions, loss of guard cell turgor and stomatal closure (37, 45). A 1 M mannitol solution has an osmotic potential of -22.6 atm (-2.3 MPa) and plasmolyzes Allium or Tradescantia cells (23). Guard cells subjected to the equivalent osmotic potential would be expected to plasmolyze and close.

Exogenous ABA increases guard cell membrane permeability, inhib-
its the operation of an active H⁺/K⁺ exchange, and causes malate leakage (50). Guard cell turgor decreases and stomata close. In tomato mutants, such as *flacca*, which have low endogenous ABA, wilting is prevented by application of exogenous ABA which causes stomatal closure (19). Stomatal apertures in epidermal strips of *Commelina communis* L. were about 3 μm after 2.5 hr of 10⁻⁴ M ABA treatment (20).

High atmospheric CO₂ increases sub-stomatal CO₂, which closes stomata (37). Raschke (36) observed the saturation kinetics of CO₂ induced closure in *Zea mays* L., and Wilson (53) observed a distinct response to atmospheric CO₂ in *C. communis*.

The environment of aseptically cultured plants differs from that of the greenhouse plants. Humidity in the jars is constant, and close to 100 % RH; light intensity is 100 to 150 μE m⁻² s⁻¹. Gas exchange and air movement is negligible because the culture jars were sealed with parafilm to prevent contamination. Intermediary metabolites, such as sucrose, inositol, and some amino acids, were available to plant through agar media. In the greenhouse, relative humidity ranged from 45 to 55 %; light intensity was usually twice that of culture jars. CO₂ and O₂ diffuse throughout the greenhouse and fans increase air exchange. Photosynthesis is the only source of substrates for intermediary metabolites in the greenhouse plants. If, as suspected, the photosynthetic rate of aseptically cultured plants was low, a constant dE/dA would be unnecessary to maintain growth. The culture jar environment did not induce development of stomatal closure mechanisms to maintain a constant gain ratio.

We hypothesize that development of functioning mature stomata
on leaves of greenhouse plants involves a) division of the guard mother cell into two closed guard cells, b) initial opening of the pore between the guard cells by $k^+$ and turgor increases (32), and c) development of a closure mechanism in response to the environment.

The greenhouse apple leaves, which had functional stomata, were aseptically cultured prior to acclimatization. Our previous study (4) showed that aseptically cultured plants acclimatized to the greenhouse within 4 to 5 days of exposure to low relative humidity. Development of the stomatal closure mechanism occurred during the period of acclimatization, possibly in response to a higher photosynthetic rate initiating a need to keep the gain ratio constant.

The major water loss during transfer of aseptically cultured plant prior to acclimatization must be stomatal and not cuticular, as previously reported (2, 46). Cuticular water loss was, however, larger from high humidity grown plants, which had less epicuticular wax, than from lower humidity plants, which had more epicuticular wax (2, 46) or wax in structured plates (16).

If the stomatal closure mechanism could be developed prior to transfer from the culture jar, more plants would acclimatize. Commercial production procedures would be more efficient. The stomatal closure mechanism might be induced by increasing the photosynthetic rate during the last stage of growth in the culture jar by lowering humidity, flowing air or $CO_2$ through the culture jar, decreasing the concentration of metabolites in the culture media, or gradually increasing light intensity.

Aseptic culture provides a controlled system for studying growth
and could be a useful system to analyze the relationships between assimilation, transpiration, and stomatal aperture. Our future studies will investigate guard cell physiology in aseptically cultured plants to determine if \( K^+ \) transport, structural differences, or other factors prevent stomatal closure.
Figure 7. Light micrographs of butyl acetate leaf imprints of greenhouse (A) and aseptically cultured (B) 'Mac 9' apple stomata taken at 0400 PST. The bar = 10 μm.
Figure 8. Percent stomatal closure plotted by time in darkness for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves. Each point represents the mean of 5 samples.
Figure 9. Percent stomatal closure plotted by time in solutions of 1 M mannitol or distilled water for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves. Each point represents the mean of 5 samples. The vertical bars represent S.D.
Figure 10. Percent stomatal closure plotted with -Log M ABA for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves treated for 1 hr. Values for control were taken from untreated leaves. Each bar represents the mean of 5 samples.
Figure 11. Percent stomatal closure plotted with 1 hr of 0.12 % atmospheric CO$_2$ treatment for greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves and with untreated controls directly from plants. Each bar represents the mean of 5 samples.
IV. Stomatal Anatomy and the Duration of Acclimatization of In Vitro 'Mac 9' Apples

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Additional index words. Malus domestica, tissue culture, aseptic culture

Abstract. Stomatal anatomy of in vitro and greenhouse acclimatized Malus domestica (Borkh.) cv. Mac 9 leaves were examined with light and scanning electron microscopy (SEM). No structural differences which could prevent closure of in vitro stomata were observed. The duration needed to acclimatize in vitro Mac 9 apples under 65% relative humidity (RH) was determined with an open-jar study up to 6 days. Percent of closed stomata were determined after a 30 min leaf drying test, and after exposure to 30 min of darkness. Transplant survival was determined after 6 weeks to assess the effects of acclimatization duration treatment. The three tests verified that 2 days of exposure to 65% RH were needed to acclimatize in vitro plants to a low RH greenhouse environment.

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2Graduate student and professor. The authors thank the Oregon Association of Nurserymen for financial assistance.
INTRODUCTION

Leaf anatomy of woody plants cultured in vitro differs from those grown at lower humidity (5, 16, 51). Differences in epicuticular wax (16), air space (5, 51), or palisade parenchyma (5, 51), however, could not explain the water stress of transferred unacclimatized in vitro plants (4).

Stomatal functioning is involved in the acclimatization of in vitro 'Mac 9' apples (4, 6). Lack of stomatal closure causes water stress of unacclimatized plants within 15 min after transfer to low RH (4, 6). Stomata of in vitro apple leaves do not close in darkness, in high osmotic pressure, with exogenous ABA application, or in high atmospheric CO₂ (6). In vitro stomata also fail to close after soaking for 24 hr in 10⁻⁶ or 10⁻⁷ M nonactin, a potassium ionophore, or after 12 hr in an etherized atmosphere which causes cell death (Appendix Tables VI, VII). Because these treatments do not close in vitro stomata but induce closure in greenhouse acclimatized stomata, differences in stomatal anatomy may be responsible for the differential response.

The objectives of this study were to determine 1) if stomatal anatomy changed during acclimatization to prevent water loss, 2) the relationship of stomatal closure to the percent transplant survival in the greenhouse, and 3) the rate of acclimatization at 65 % RH.

MATERIALS AND METHODS

'Mac 9' apple plants were cultured in vitro (8) by Microplant Nursery of Gervais, Oregon. Culture jars of rooted plants were placed for 2 months in 22°C under 16 hr photoperiod of 100 μE m⁻² s⁻¹
of cool white fluorescent light prior to treatment. Greenhouse acclimatized plants were cultured in vitro, transferred to the greenhouse, and grown for 12 months in peat:perlite (v/v) under natural photoperiod at 18 to 24°C.

An open jar acclimatization study similar to that of Brainerd and Fuchigami (4) was conducted from June 13 to 19, 1981. Culture jar lids were removed for 0, 2, 4, or 6 days. Ten ml of water was added daily to the open jars to moisten the agar. The RH in the culture room was maintained at 65 ± 5% by a Sears Rotobelt Console Evaporative Cooler.

Two jars, each containing 10 plants, were randomly allocated to each open jar treatment time. Nine leaves were excised from 2 plants in each jar; 2 for a 20 min drying study, 2 for a 30 min darkness study, and 5 for light microscopy and SEM. Eight plants in each open jar treatment were used in a greenhouse transplant study.

For the light microscopy and SEM, leaves were fixed in formalin-aceto-alcohol, embedded in paraffin, sectioned, and stained in safranin and fast-green (21). Stained leaf sections were then coated with 200 Å gold and examined in an ISI MSM-2 scanning electron microscope at 30° angle and 15 KV.

In the drying study, excised treated leaves were placed abaxial side-up on a lab bench for 30 min at 40% RH. Silicon-rubber (Dow-Corning compound 3110 RTV) and butyl acetate leaf imprints were made and microscopically examined to determine stomatal closure. The number of closed stomata (less than 2 μm aperture) from the first 100 counted on each leaf imprint determined the percent of closed
 stomata (4).

In the dark study, the open jars were placed in a photographic darkroom for 30 min. Leaf imprints were made from excised leaves and percents of closed stomata determined for each imprint. Mean differences were detected by analysis of variance and separated by LSD, 1 %.

For the transplant study, plants from the open jar treatments were placed in pots of peat:perlite (v/v) directly on a greenhouse bench; no high humidity chamber was used. The greenhouse temperature was 18 to 24°, 30 to 40 % RH, and under a natural photoperiod. After 6 weeks, the percent transplant survival (the number of live plants per 8 plants transplanted, X 100) was calculated. Plant height, from the soil line to the most apical node was measured and plant quality was evaluated. Analysis of variance was calculated on plant height.

RESULTS AND DISCUSSION

The stomatal anatomy of in vitro, open jar treated, and greenhouse acclimatized 'Mac 9' leaves was similar. Cross sections of guard cells of all treatments, viewed in the light microscope, had thick, darkly staining inner walls, and darkly staining cytoplasm. Discernible pores of 0, 2, 4, 6 days, and of the greenhouse acclimatized treatments had apertures greater than 2 μm, i.e., were open, although leaves were dehydrated by the t-butyl alcohol series. Scanning electron micrographs also showed similar stomatal anatomy between in vitro and greenhouse plant leaves (Fig. 12). A stomatal lip was present on the outer wall of both types of stomata (Fig. 12).
12 B-F). Thick inner walls were present in both (Fig. 12C, 12E, arrows). Guard cell lengths were equivalent, and guard cell lumens were similar in size and shape (Fig. 12).

Raschke (38) cautioned that accurate observations on stomatal anatomy are difficult to make. Guard cell walls are hydrophilic, elastic in directions normal to the paradermal plane, and have high cation exchange capacities. "Preparation for conventional electron microscopy causes serious distortions of guard cell walls" (38). Our samples were fixed and dehydrated by conventional light microscope techniques prior to SEM preparation. Our evaluation may not accurately represent that of living leaves because the dehydration process changes cell wall thickness and lumen size and shape. In *vitro* guard cell walls, developing under high humidity and in nutrient media, may contain more water and more ions than those of lower RH plants growing in soil. High apoplastic water and ion content in *in vitro* guard cell walls may help prevent stomatal closure.

The percent of closed stomata was significantly higher (Table 3) in the 30 min dry test and the 30 min dark test for 2 or more days of open jar treatment than for 0 days (*in vitro* plants). The percent transplant survival also increased from the Day 0 to the Day 2 open jar treatment, from 50 to 100 % (Table 3). These results indicated that *in vitro* plants adjusted to a low RH greenhouse environment, i.e., acclimatized, within 2 days of exposure to 65 % RH.

In our previous study (4), *in vitro* plants acclimatized after four to five days of exposure to 30 to 40 % RH (4), whereas, in this study plants acclimatized within two days of exposure to 65 % RH.
(Table 3). This time difference could be due to RH. We conclude that the duration of acclimatization was altered by several days by changing the RH of the acclimatizing environment.

*In vitro* plants were acclimatized by removing jar lids for a period of time in a growth chamber. Lid removal allowed leaves to acclimatize to low humidity prior to disrupting roots from agar. Agar moisture was maintained and plants were removed from open jars within 6 days, prior to formation of contamination. The duration of open jar, from 2 to 6 days, did not influence the plant height (Table 3) or quality of surviving transplants.

In summary, stomatal anatomy, as viewed through light and SEM, was unchanged throughout leaf acclimatization to low humidity. The percent of transplant survival was related to the stomatal closure ability of acclimatized *in vitro* plants. A 30 min dry test and a 30 min dark test examining the percent of closed stomata, and the percent transplant survival after 6 weeks, verified that 2 days of 65% RH were sufficient to acclimatize *in vitro* plants to a low RH greenhouse environment.
Table 3. Percent of closed stomata after 30 min of drying or darkness, percent of transplant survival, and plant height 6 weeks after transplanting for in vitro 'Mac 9' apples with jar lids removed for 0, 2, 4, or 6 days.

<table>
<thead>
<tr>
<th>Time of open jar days</th>
<th>30 min drying %</th>
<th>30 min in dark %</th>
<th>% transplant survival</th>
<th>Plant Height cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.5 A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0 A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>50</td>
<td>6.9NS&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>84.7 B</td>
<td>41.5 B</td>
<td>100</td>
<td>7.1NS</td>
</tr>
<tr>
<td>4</td>
<td>78.7 B</td>
<td>54.0 B</td>
<td>100</td>
<td>7.6NS</td>
</tr>
<tr>
<td>6</td>
<td>92.5 B</td>
<td>81.7 C</td>
<td>75</td>
<td>7.8NS</td>
</tr>
</tbody>
</table>

<sup>2</sup>Each value is the mean of 4 leaf samples. LSD, 1% level, separated mean differences in the columns.

<sup>y</sup>Calculated from 8 plants per treatment, 6 weeks after transplanting to the greenhouse.
Figure 12. A. Approximate cutting planes of the SEM micrographs in 12 B-F. B. Greenhouse stoma cut in plane aa'. C. Greenhouse stoma cut in plane bb'. Arrow points to inner wall thickening present on majority of guard cell cross sections. D. Almost complete in vitro stoma. E. In vitro stoma cut in plane cc'; o, i, d, v denote outer, inner, dorsal, and ventral guard cell walls. Arrow points to inner wall thickening. F. In vitro stoma cut in plane bb'. 


APPENDIX
Appendix Table I. Analyses of variance for upper and lower epidermal cells and palisade parenchyma for aseptically cultured, greenhouse transferred, and field grown 'Pixy' plums. LSD calculated at 1% level.

<table>
<thead>
<tr>
<th>Source</th>
<th>S.S.</th>
<th>df</th>
<th>M.S.</th>
<th>F</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upper Epidermis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1053</td>
<td>74</td>
<td>582</td>
<td>2</td>
<td>470</td>
</tr>
<tr>
<td>Plant type</td>
<td>291.4</td>
<td>44.6</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>6.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lower Epidermis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>643</td>
<td>74</td>
<td>57.6</td>
<td>2</td>
<td>585</td>
</tr>
<tr>
<td>Plant type</td>
<td>28.8</td>
<td>3.5</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>8.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Palisade Parenchyma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47684</td>
<td>74</td>
<td>45122</td>
<td>2</td>
<td>5262</td>
</tr>
<tr>
<td>Plant type</td>
<td>22561.0</td>
<td>634</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>35.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data were collected assuming that each cell type was from a different population and a one-way analysis of variance was calculated for each type. To check interactive effects between cell types, a two-way AOV should have been calculated.
Appendix Table II. Friedman's test (28) and a multiple comparison technique based on Friedman's ranked sums (18) for percent air space of aseptically cultured, greenhouse, and field grown 'Pixy' plum.

<table>
<thead>
<tr>
<th></th>
<th>Mean % air space</th>
<th>Rank Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aseptically cultured</td>
<td>20.6</td>
<td>10</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>13.3</td>
<td>21</td>
</tr>
<tr>
<td>Field Grown</td>
<td>9.55</td>
<td>29</td>
</tr>
</tbody>
</table>

\[ H \text{ (sum of squares of rank totals)} = 1382^{**} \]

\[ q \text{ (1 \% critical value for comparing rank sums)} = 9.21 \]
Appendix Table III. Analysis of variance of the relative water content (RWC) for leaves 1 hr after excision from aseptically cultured 'Mac 9' apples exposed to 30 to 40 % RH for 0, 6, 12, 24, 48, 96 hr and from the greenhouse.

<table>
<thead>
<tr>
<th>Source</th>
<th>S.S</th>
<th>df</th>
<th>M.S.</th>
<th>F</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>3942</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>2912</td>
<td>6</td>
<td>485</td>
<td>13</td>
<td>9.69</td>
</tr>
<tr>
<td>time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>1030</td>
<td>28</td>
<td>36.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix Table IV. Analysis of variance for a multiple regression equation to test the significance of percent stomatal closure (%SC) and constant water loss (CWL) after the effect of the other variable has been removed (44).

<table>
<thead>
<tr>
<th>Source</th>
<th>S.S.</th>
<th>df</th>
<th>M.S.</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>% SC and CWL</td>
<td>47.8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% SC alone</td>
<td>0.295</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWL after % SC removed</td>
<td>47.505</td>
<td>1</td>
<td>47.505</td>
<td>339.2**z</td>
</tr>
<tr>
<td>% SC and CWL</td>
<td>47.8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWL alone</td>
<td>1.135</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% SC after CWL removed</td>
<td>46.665</td>
<td>1</td>
<td>46.665</td>
<td>333.2**z</td>
</tr>
<tr>
<td>Deviation</td>
<td>3.08</td>
<td>22</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>

"Conclusion: Both coefficients, percent stomatal closure and constant water loss, contribute significantly to the rate of water loss for aseptically cultured and greenhouse 'Mac 9' apple leaves."
Appendix Table V. Analyses of variance for percent of closed stomata after 30 min drying, 30 min of darkness, and plant height 6 weeks after transplanting for \textit{in vitro} 'Mac 9' apples with jar lids removed for 0, 2, 4, or 6 days.

<table>
<thead>
<tr>
<th>Source</th>
<th>S.S.</th>
<th>df</th>
<th>M.S.</th>
<th>F</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min drying</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>21581</td>
<td>15</td>
<td>1458</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day effect</td>
<td>20964</td>
<td>3</td>
<td>6988</td>
<td>135**</td>
<td>15.5</td>
</tr>
<tr>
<td>Error</td>
<td>617.5</td>
<td>12</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min darkness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14743</td>
<td>15</td>
<td>983</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day effect</td>
<td>13868</td>
<td>3</td>
<td>4622</td>
<td>63**</td>
<td>18.5</td>
</tr>
<tr>
<td>Error</td>
<td>876</td>
<td>12</td>
<td>73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant Height (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>622</td>
<td>31</td>
<td>20.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day effect</td>
<td>88</td>
<td>3</td>
<td>29.3</td>
<td>1.53NS</td>
<td>---</td>
</tr>
<tr>
<td>Error</td>
<td>534</td>
<td>28</td>
<td>19.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix Table VI. Percent of closed stomata\textsuperscript{z} for excised in vitro and greenhouse 'Mac 9' apple leaves treated for 24 hr in distilled water, $10^{-6}$ M nonactin\textsuperscript{y}, and $10^{-7}$ M nonactin\textsuperscript{x}.

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Leaf replicate</th>
<th>Distilled \textit{water}</th>
<th>$10^{-6}$M</th>
<th>$10^{-7}$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{in vitro}</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{greenhouse}</td>
<td>1</td>
<td>17</td>
<td>75</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>78</td>
<td>96</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>97</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>27</td>
<td>94</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>94</td>
<td>99</td>
<td>94</td>
</tr>
</tbody>
</table>

\textsuperscript{2}Percent closed stomata determined by number of stomata with apertures less than 2 $\mu$m out of the first 100 stomata observed per leaf sample.

\textsuperscript{y}$10^{-6}$ M nonactin in 1% EtOH.

\textsuperscript{x}$10^{-7}$ M nonactin in 0.1% EtOH.
Appendix Table VII. Percent of closed stomata\textsuperscript{2} for excised \textit{in vitro} and greenhouse 'Mac 9' apple leaves treated for 12 hr in a closed etherized atmosphere and untreated (stomatal aperture counted upon excision from plant).

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Leaf replicate</th>
<th>Ether</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{in vitro}</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>greenhouse</td>
<td>1</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{2}Percent closed stomata determined by the number of stomata with apertures less than 2 \textmu m out of the first 100 stomata observed per leaf sample.
Appendix Table VIII. Percent of closed stomata\(^z\) for excised \textit{in vitro} and greenhouse 'Mac 9' apple leaves frozen at \(-40^\circ\text{C}\) for 18 hr and thawed at \(24^\circ\text{C}\) for 6 hr prior to making leaf imprints.

<table>
<thead>
<tr>
<th>Leaf replicate</th>
<th>Greenhouse</th>
<th>In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plant number</td>
<td>Plant Number</td>
</tr>
<tr>
<td></td>
<td>cntr(^y) 1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>84</td>
</tr>
</tbody>
</table>

\(^z\)Percent of closed stomata determined by the number of stomata with apertures less than 2 \(\mu\text{m}\) out of the first 100 stomata observed per leaf imprint.

\(^y\)Control imprints were made on untreated leaves directly after excision.