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Sorbitol-induced osmotic stress (SIOS) to the root was found to be effective in reducing transpirational water loss in plants during water stress. Mechanisms of SIOS in stomatal control were investigated in containerized apples (*Malus domestica* Borkh. 'Gala'/M26) when leaf water potential (Ψ_w), osmotic potential (Ψ_s), turgor potential (Ψ_p), stomatal conductance (g_s), relative water content (RWC), and abscisic acid (ABA) content of the leaf and xylem sap were determined. Sorbitol (-1.2 MPa) was applied as a soil drench. For ABA determination, crude leaf extract and xylem sap were used for the radioimmunoassay method.

Stomatal conductance decreased rapidly within one hr after SIOS treatment, which was closely associated with a decline in leaf water status (Ψ_w, Ψ_p, and RWC). The SIOS-induced earlier reduction in g_s caused plants to resist greater desiccation stress than the untreated controls.

Leaf ABA production was stimulated by SIOS treatment, but the effect differed between plants adapted in greenhouse and outdoor conditions. In greenhouse-adapted

plants, SIOS caused leaf ABA to increase significantly two hrs after treatment, but had no effect in outdoor-adapted plants. However, g_s was similarly reduced in both conditions by about 55%. This difference in leaf ABA production between plants adapted in both conditions could be associated with the concurrent differential decrease in Ψ_p; baseline Ψ_p was much higher in the outdoor-adapted plants than in the greenhouse-adapted plants. In the greenhouse-adapted plants, Ψ_p decreased by 61% (from 1.15 to 0.45 MPa) within two hrs after SIOS treatment, whereas Ψ_p decreased by 27% (from 2.15 to 1.58 MPa) in the outdoor-adapted plants.

Xylem sap ABA, measured two hrs after SIOS treatment from the outdoor-adapted plants, was not affected, whereas g_s and leaf Ψ_w (thus Ψ_p) significantly decreased. Significant increase in ABA content in the xylem sap and leaf, however, occurred 1 and 3 days after treatment, respectively. ABA level and g_s data suggested that the immediate reduction in g_s by SIOS treatment may have resulted from hydraulic effects on g_s, rather than chemical effects (e.g., root ABA signal), at least for the outdoor-adapted plants.

WATER RELATIONS, STOMATAL CONDUCTANCE, AND ABSCISIC ACID CONTENT OF CONTAINER-GROWN APPLE (*Malus domestica* Borkh.) PLANTS IN RESPONSE TO SORBITOL-INDUCED OSMOTIC STRESS

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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LIST OF ABBREVIATIONS

[ABA] (abscisic acid concentration)

Ψ_s (leaf osmotic potential)

PAR (photosynthetically active radiation)

PEG (polyethylene glycol)

RIA (radioimmunoassay)

RWC (relative water content)

SIOS (sorbitol-induced osmotic stress)

g_s (stomatal conductance)

Ψ_w (total leaf water potential)

Ψ_p (turgor potential)

θ (volumetric soil water content)

WATER RELATIONS, STOMATAL CONDUCTANCE, AND ABSCISIC ACID CONTENT OF CONTAINER-GROWN APPLE (*Malus domestica* Borkh.) PLANTS IN RESPONSE TO SORBITOL-INDUCED OSMOTIC STRESS

CHAPTER 1

INTRODUCTION

Water is the most important compound worldwide for sustaining life. More than twenty nations are now facing chronic water shortage, and the number will surely increase with increasing demands of water for agriculture, industry in general and for human consumption. In the cultivation of plants much of the water applied is lost either as run-off, evaporation and/or transpiration. Among the plant producers nurserymen that grow plants in containers are among the heaviest users of water. Because of the limited volume of the container, plants grown in containers require frequent irrigation to maintain the water status of the plant growing medium. Furthermore, because of the shape, size and surface area of the container, irrigation practices to direct the water into the container without loss either between or through the container is generally inefficient and large quantities of water are wasted. For example, aerial application (such as sprinkler irrigation) of water and agricultural production chemicals is inefficient because a large quantity misses the individual, spaced containers (Green, 1990). Considering the inefficient water use and the potential for contamination of groundwater through leaching of the fertilizers and other chemicals in the containers, developing methods to reduce water loss of container grown plants will be obviously beneficial to nurserymen and the environment in general.

Plants respond to water stress either by postponing dehydration while maintaining a high plant water potential (i.e., drought avoidance), or by tolerating dehydration with survival at low plant water potential (i.e., drought tolerance) (Kramer, 1983; Turner, 1986). The primary way plants regulate water flow through the soil-plant-atmosphere continuum over the short term is by regulating transpiration through stomata. When transpiration exceeds water absorption, water stresses develop in plants and growth is reduced (Kramer and Boyer, 1995).

There are two basic methods of reducing water loss through the stomata to increase desiccation resistance. Firstly, water loss may be reduced by physically plugging up the stomata with a surface applied large organic polymer (film-forming antitranspirants) (Murakami et al., 1990; Englert, 1992; Remmick, 1995). Film-forming antitranspirants are often difficult to apply, and it is difficult to obtain complete coverage; they are often toxic to the leaf and young stem tissues. The results are often specific to the plant and environmental conditions (Englert, 1992). Secondly, water loss can be reduced by introducing compounds into the plant and causing the stomata to close physiologically. Abscisic acid (ABA) has been effective in reducing transpiration in a wide range of species (Davies and Mansfield, 1983). However, it is expensive to synthesize, rapidly inactivated by sunlight and broken down by plants (Davies and Mansfield, 1983). In addition, its effects have been often temporary and/or inconsistent.

A need therefore exists for developing a plant water loss reducing agent which is non-toxic, environmentally acceptable, and relatively inexpensive. Recently, Great Lakes Chemical Corporation (West Lafayette, IN) reported the use of GLK8924 as a soil drench to reduce water loss in several herbaceous plants (C.H. Shin, personal communication).

Because of our interest in use of antitranspirants, we were approached by scientists in the Great Lakes Chemical Corporation to conduct further tests of this compound on several plant species. During these tests the disclosure of GLK8924's formulation was not available because of the patenting process by Great Lakes Chemical Corp. GLK8924 has since been granted a U.S. Patent (No. 5459121) on 17 October 1995. The active ingredient of this compound is sorbitol. The compound has been cleared by the Environmental Protection Agency for use as an antitranspirant for plants, and is also known to be effective for conditioning plants to withstand water stress.

Sorbitol has often been used as a metabolically inert osmoticum (Paleg and Aspinall, 1981; Thompson et al., 1986; Bowman, 1988, Auge et al., 1992). The structure (with six hydroxyl groups) is such that, in the absence of a specific transport system, it crosses the plasmalemma very slowly, thus satisfying one suitability requirement (Cram, 1984). Bowman (1988) and Auge et al. (1992) reported that lowering soil water potential with sorbitol solution significantly decreased stomatal conductance in *Andropogon glomeratus* and *Vigna unguiculata*, respectively. However, mechanisms of sorbitol-induced osmotic stress in the initial decline in stomatal conductance have never been studied anywhere.

Water stress can cause a rapid accumulation of ABA in many plants, including apple (Wright, 1969; Walton, 1980; Robinson and Barritt, 1990). This results from *de novo* synthesis rather than release from a bound form of ABA, as in red kidney bean (Pierce and Raschke, 1980) and Scotch pine (Hogue et al., 1983). Davies and Lakso (1983) and Robinson and Barritt (1990) observed in mature apple leaves ABA accumulation as a response to PEG (polyethylene glycol)-induced water stress, and

concluded that the increases in ABA are correlated with cell turgor pressure. The increases in ABA cause stomata to close (Jones and Mansfield, 1970; Wright, 1977).

There is no doubt that plants in drying soil experience a decrease in bulk leaf water potential and leaf turgor with a consequent reduction in stomatal conductance and growth. However, roots in drying soil can affect shoot physiology independently of shoot water potential (Bates and Hall, 1981; Livingston and Black, 1987; Grantz, 1990; Saliendra et al., 1995). Over the last decade, analyses have served mainly to invalidate the theory of stomatal control by leaf water status alone and to demonstrate the controlling effect of chemical messages from roots to shoots (e.g. Davies and Zhang, 1991). Despite this, it is difficult to overlook consistent evidence, from the past 20 years, of a correlation between stomatal behavior and leaf water status. Tardieu and Davies (1992 and 1993) reported that leaf water potential could have a role in the control of stomatal sensitivity to ABA.

Most of the work examining drought-induced stomatal closure has been done with herbaceous species. In cowpea (Bates and Hall, 1981), in wheat (Gollan et al., 1986), in sunflowers (Zhang and Davies, 1989a, 1989b, and 1990), and maize (Zhang and Davies, 1991), a close relationship between ABA in the xylem sap and stomatal conductance was observed. In one of the few studies involving woody plants, Khalil and Grace (1993) reported that stomatal conductance in sycamore maple seedlings (*Acer pseudoplatanus* L.) was related to ABA concentration in the xylem sap, with no significant perturbation in shoot water status. The increase in root ABA was associated with the increase in xylem sap ABA. Henson and Turner (1991) showed that the level of ABA in the leaf was proportional to that produced by the roots of three lupin species.

In contrast, Saliendra et al. (1995) suggested that in *Betula occidentalis* seedlings, it was hydraulic signals that probably prompted stomatal control against water stress, rather than chemical messengers (e.g., ABA) originating in the roots. Any treatment or event that changes soil water potential or hydraulic conductance results in hydraulic signals from the roots, which provides a rapid means of communication within the plant (Malone, 1993; Whitehead et al., 1996). Stomatal conductance was positively correlated with the apparent hydraulic conductance of the soil-root-shoot pathway in woody species (Kuppers, 1984) and in sugarcane (Meinzer and Grantz, 1990). Malone (1993), Tardieu and Davies (1993), and Whitehead et al. (1996) suggested that the rapid transmission of a hydraulic change may trigger the generation of a chemical signal in the leaves to regulate stomatal conductance.

The overall objective of this study was to examine mechanisms of sorbitol-induced osmotic stress (SIOS) in reducing transpirational water loss in container-grown apple plants during water stress. This study was divided into the following objectives: 1) Determine the effects of SIOS on water relations (water potential, osmotic potential, turgor potential, and relative water content) and their relationship to stomatal conductance; 2) Determine the effects of SIOS on bulk leaf ABA concentration and its relationship to stomatal conductance; 3) Determine the effects of SIOS on ABA concentrations in the leaf and xylem sap, and their relationship to leaf water status and stomatal conductance; and 4) Determine comparative effects of sorbitol, polyethylene glycol, and sodium chloride on water relations, gas exchange, and ABA production in the leaf and xylem sap of apple. Objectives 1 and 2 were studied with greenhouse-adapted plants. Objectives 3 and 4 were studied on outdoor-adapted plants.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Importance of Water

Water is one of the most abundant and important substances on the earth's surface, but about one third of the world's potentially arable land lacks an adequate water supply (Kramer and Boyer, 1995). Although agriculture receives much of its water from rainfall, farmers are still major consumers of stored water, spending billions of dollars annually on irrigation systems to alleviate water shortages and improve crop yields (Kramer and Boyer, 1995). Availability and cost of fresh water for agriculture is becoming a major issue because of rapidly increasing population and industrial and recreational needs (Simpson, 1981).

Water constitutes about 80 to 90% of the fresh weight of most herbaceous plant organs and over 50% of the fresh weight of woody portions (Karamanos, 1981). Water functions in plants as a hydraulic agent in maintaining turgor and enhancing expansion growth and a biochemical reactant in photosynthesis and other important metabolic reactions. McIntyre (1987) suggested that water be regarded as the major factor in plant growth regulation. Water is also ecologically important because the distribution of plants over the earth's surface is determined chiefly by water and temperature (Thornthwaite and Marther, 1957; Kozlowski, 1968).

2.2 Plant Water Status

Plant water status is the quantification of the condition of water relative to its requirement (Spomer, 1985). According to Taylor (1968) and Kramer (1972), plant water status is best characterized by its physicochemical availability (e.g., water potential) for plant functions, amount present (e.g., water content or relative water content), and movement through the system. Physicochemical availability relates to absolute availability or energy status, whereas the amount plays subtle roles in maintaining functional structure (Boyer, 1969; Nobel, 1991).

Water potential (Ψ) is defined by its chemical potential (μ) relative to a standard reference (pure, free water at a specified temperature and pressure) that has a value of zero (100% available) (Nobel, 1991). Ψ determines the tendency for net water movement within the system (Kramer et al., 1966; Rose, 1966; Taylor, 1968; Nobel, 1991, Kramer and Boyer, 1995). Water content, the amount in the system potentially available for plant functions, is determined by tissue water-holding characteristics and the balance between water movement in and out (Spomer, 1985). Water movement that determines tissue water balance and material transport within the plant is largely controlled by soil and atmospheric environments which determine the supply (absorption) and demand (transpiration) (Slatyr and Gardner, 1965; Slavik, 1974).

Water potential is usually measured as the vapor pressure difference (VPD) between water in the plant and in a reference: $\Psi = [RT \cdot \ln(e/e^o)] \cdot v^{-1}$, where R is the universal gas constant, e is the system water vapor pressure, e^o is the reference water vapor pressure, and T is the temperature in degrees Kelvin (Spomer, 1985; Nobel, 1991).

To measure and describe water potential, pressure (MPa or bar), rather than energy concepts, is traditionally used and is expressed as the chemical potential difference on a partial molal volume (v) of water basis ($cm^3 \cdot mole^{-1}$): $\Psi = (\mu - \mu_o) \cdot v^{-1}$, in $ergs \cdot cm^{-3}$ or $Joules \cdot cm^{-3}$ (Nobel, 1991).

Any factor decreasing water potential also decreases the physicochemical availability. The total water potential (Ψ_w) of any particular system is an integrated sum of various components, including osmotic (or solute; Ψ_s), pressure (or turgor; Ψ_p), matrix (Ψ_m), and gravitational (Ψ_g) potentials:

$$\Psi_w = \Psi_s + \Psi_p + \Psi_m + \Psi_g \text{ (Nobel, 1991).}$$

Ψ_s, a negative value, depends on the concentration of solutes in the cell. Solutes lower Ψ_s and provide the driving force for water uptake. Ψ_s in a solution depends on the total number of solute particles (molecules or ions), rather than on their kind or charges (Nobel, 1991).

Ψ_p, a positive value, measures the difference between internal and external pressure. Excess pressure inside the cells drives cell expansion and affects processes such as stomatal movement. Ψ_p is perhaps a better indicator of water stress, especially for turgor-dependent processes such as growth (Hsiao et al., 1976).

Ψ_m results from the binding of water molecules to protoplasmic and cell wall constituents. In most growing plants, tissues with mature, vacuolated cells have a Ψ_m of only about -0.01 MPa (Wiebe, 1966). Therefore, Ψ_m in fresh tissue is often discounted because it contributes little to total Ψ_w.

Ψ_g depends on gravitational force on the cell solution. It decreases by only 0.01 MPa for each meter gain in height (Jones et al., 1985) and differs about 0.03 MPa from top and bottom in a 3-meter-tall tree. Ψ_g is usually negligible for short plants.

Because Ψ_m and Ψ_g are tenuous, in physiologically active plant tissues, the useful equation for total water potential becomes: $\Psi_w = \Psi_s + \Psi_p$. Rose (1966) and Kramer and Boyer (1995) also claimed that the most important water potential components in horticultural plants were Ψ_s and Ψ_p, and these components largely determined tissue water relations.

Ψ_w is considered by most plant physiologists to be the most useful and significant means to describe plant water status. However, Ψ_w is not necessarily a good indicator of water stress because plant responses are only indirectly relevant to water flow (Hsiao et al., 1976). A transpiring plant has gradients of Ψ_w between tissues, making it difficult to measure and define plant water status. Therefore, the relatively stable predawn Ψ_w, which can estimate soil Ψ, is often used (Jones et al., 1985).

2.3 Plant Tissue Water Status Measurement

2.3.1 Water content measurement

Plant water content, the difference between the plant fresh (m_f) and dry (m_d) mass, is the most common water status measurement because it is simple and straightforward.

Barrs (1968) cautioned that the expression of water content alone was impractical because it could not be compared with other water content measurements on the same or

other plants. A common frame of quantitative reference or base was required. Tissue fresh mass (Halevy and Monselise, 1963; Barrs, 1968), dry mass (Barrs, 1968), and turgid mass (Hewlett and Kramer, 1963; Barrs, 1968) are widely used bases for expressing water content quantitatively.

Spomer (1985) and Boyer (1995) showed that relative water content (RWC), using a turgid mass base, was the most widely accepted expression of plant tissue water content. RWC = $(m_f - m_d)/(m_t - m_d)$, where m_t is turgid mass. RWC indicates water content relative to the maximum possible (100% relative turgidity). Catsky (1965) said that using RWC was limited because it was difficult to achieve accurate turgid water content measurements. Several procedures have been developed, each of which may provide different values for identical tissue samples. Procedures include floating or immersing, or inserting tissues in water until their mass remains constant.

Respiratory decrease in dry mass can also cause significant error in determining RWC, especially during long immersion periods (Catsky, 1965; Rains et al., 1980). Methods to minimize this source of error have included reduced temperatures (Catsky, 1969), chemical inhibition (Slatyr and Barrs, 1965), and photosynthetic light (Rains et al., 1980). Solute leakage from the sample over long rehydration periods also may decrease dry mass (Gates, 1968; Boyer, 1995).

2.3.2 Water potential measurement

Ψ_w usually describes the tendency for water to move between the observed system and the reference across a barrier permeable only to water (Spomer, 1985; Nobel, 1991). No net movement occurs at equilibrium (uniform Ψ_w). Ψ_s is measured after eliminating

the turgor components by disrupting the cell membranes (Spomer, 1985; Boyer, 1995) or by measuring the water potential of expressed cell sap (Slavik, 1959; Boyer, 1995). The turgor component is obtained indirectly as the difference between Ψ_w and Ψ_s (Rose, 1966) or directly by pressure probes (Green and Stanton, 1967).

Rawlins (1972) stated that the thermocouple psychrometer (with a sensitive wet-dry bulb) was the most commonly used and probably most accurate vapor phase method for water potential measurement. The theory and practice of this technique has been reviewed extensively (Barrs, 1968; Boyer, 1995). Briefly, water potential measurements are inferred from measurements of equilibrium relative humidity (water vapor pressure, assumed to be a direct function of sample water potential) in a small, sealed chamber containing sample and reference. The psychrometer is calibrated with a series of solutions of known potential (Meyn and White, 1972). This technique is very sensitive, so precautions should be taken to avoid temperature fluctuations of more than a few degrees Celsius (Koorevaar and Janse, 1972; Spomer, 1985).

The pressure chamber has been widely used to study plant water relations (Tyree and Hummel, 1972; Ritchie and Hinckley, 1975; Tyree and Jarvis, 1982; Turner, 1988). The theory and use of this instrument has been thoroughly reviewed by Barrs (1968), Slavik (1974), and Boyer (1995). In its simplest application, it is used to estimate the water potential of a plant organ. This is achieved by excising an organ, such as a leaf or shoot, and sealing the sample in a pressure vessel, with the cut surface protruding just outside the seal. When increasing gas pressure is applied on the sample inside the chamber, cell sap water potential correspondingly increases and forces water back into

the xylem. When xylem sap just returns to the cut surface, the applied pressure equals that of the sap in the xylem vessels exposed to atmospheric pressure. The pressure reading at this point is related to leaf water potential by $\Psi = P + \Psi_o^{xylem}$ (Scholander et al., 1964), where P is the negative pressure component of xylem sap water potential and Ψ_o^{xylem} is the negative osmotic effect of solutes in the xylem sap. P and Ψ_o^{xylem} represent forces in the vascular system that tend to remove water from leaf cells. At the pressure balance point, values of P and Ψ_o^{xylem} are opposing and equal in magnitude to cell total water potential, and no flow occurs. Water potential can be estimated without accounting for Ψ_o^{xylem}, because xylem sap osmotic potential is generally greater than –0.3 MPa. However, accurate determinations require measurements of Ψ_o^{xylem} (Ritchie and Hinckley, 1975).

The pressure chamber is presently the simplest, most rapid method available for estimating leaf Ψ_w and perhaps is the only practical field method (Boyer, 1995). It also can be used to estimate leaf Ψ_s and Ψ_m (Boyer, 1967). Destructive sampling, unfortunately, prevents repeated measurements of the same organ. The pressure chamber values usually parallel those of the psychrometer (Spomer, 1985; Pomper, 1995), but the accuracy of pressure chamber measurements varies among species (Ritchie and Hinckley, 1975; Boyer, 1969 and 1995).

2.4 Plant Water Transport and Water Stress Development

Water in the xylem sap is almost always conducted under tension. Dixon (1914) first proposed that the cohesive properties of water allowed the propagation of negative pressure (physically equivalent to a tension or a pulling force) from the leaves to soil

water. These tensions are created as water retreats into minute pores within the matrix of cellulose fibers of the leaf cell walls during evapotranspiration.

Capillary forces maintain a negative Ψ_m, which is balanced by tension in the water within xylem elements. This tension forms a water potential gradient that is propagated down the stem to the roots. The gradient allows roots to take up soil water.

Movement of water in the xylem depends on the continuity of the water column. If it is broken anywhere between the roots and leaves, the propagation of tension (hence the flow) is arrested (Tyree and Sperry, 1988).

Plant hydrostatic pressures are normally between -1 and -2 MPa, although much lower pressures are common in desert plants and mangroves (Tyree and Sperry, 1988). Evolution of higher tensions is normally prevented by stomatal regulation (Tyree and Sperry, 1988; Sperry et al., 1993). However, when stomatal control is no longer sufficient to check stress development, breaks in the xylem continuity, or cavitation events, can occur (Zimmermann, 1983; Tyree and Sperry, 1988).

Unless a cavitated element is quickly refilled, embolism develops and the element becomes filled with air diffused from surrounding tissue (Sperry and Tyree, 1988). The hydraulic conductance of the stem is then reduced, creating greater resistance to water flux. Embolism can be induced by desiccating (or droughting) excised twigs (Sperry and Tyree, 1988) or entire seedlings (Tyree and Dixon, 1986; Borghetti et al., 1989; Tyree at al., 1992). It can also arise during winter cycles of freezing and thawing.

Xylem failure is recognized as a major factor in determining plant distribution and adaptation to habitats (Tyree and Sperry, 1989; Tyree and Ewers, 1991). Species differ in

their vulnerability to cavitation; xylem failure can limit growth and productivity and even result in dieback or death. Sperry and Pockman (1993) experimented (e.g., direct air-seeding) with reducing hydraulic conductance of stems of intact *Betula* plants. They showed that if stomatal regulation was unable to control transpirational water loss to meet the reduced sap flux, cavitation began in the surrounding xylem cells, causing nearly complete embolism, and resulting in death for tissues distal to injected stems.

2.5 Effects of Water Stress

2.5.1 Crop yield

The worldwide yield loss from water deficits probably exceeds the loss from all other causes combined (Kramer, 1980). The level of yield reduction depends on the degree, duration, and timing of the deficit. Anderson and White (1974) reported that lack of irrigation reduced total biomass of green peas by 47%, but the yield was decreased by only 36%. However, in maize, although drought stress during the grain-fill period reduced total dry matter by only 30%, there was a 47% reduction in grain yield (Downey, 1971). In wheat total dry matter decreased by 52%, but grain yield was 70% lower, when water deficit was imposed five weeks before ear emergence (Beggs and Turner, 1976).

Growth and development of trees in a particular year is often affected by conditions in the previous season. Water stress influences not only economic yield but yield quality. Proebsting and Middleton (1980) found that in peaches and pears water deficits caused flower bud differentiation, decreased fruit growth, and altered fruit astringency.

Table 2-1. Generalized sensitivity of plant processes to water stress (Based on Hsiao et al., 1976).

Process Affected	Reaction	Reduction[x] in Ψ_w that affects the process (MPa) 0	1	2
Cell growth	–[y]	----------[z]		
Protein synthesis	–	----------		
Nitrate reductase	–	----------		
ABA synthesis	+	----------		
Stomatal opening	–			
a) Mesophytes		----------		
b) Some xerophytes			----------	
CO_2 assimilation	–			
a) Mesophytes		----------		
b) Some xerophytes			----------	
Respiration	–	----------		
Proline synthesis	+		----------	
Sugar level	+		----------	

[z] Length of the line represents the range of stress levels within which a process first is affected.

[y] Water stress causes an increase (+) and decrease (-) in the process.

[x] The reduction in Ψ_w is compared with Ψ_w of well-watered plants under mild evaporative demand.

2.5.2 Physiological processes

Sensitivities of several plant processes to stress were summarized by Hsiao et al. (1976) (Table 2.1). One of the most sensitive processes is cell enlargement, most importantly, a marked reduction in leaf area. Boyer (1970) found that the rate of maize leaf enlargement declined rapidly at leaf Ψ_w below -0.2 MPa and ceased at -0.7 to -0.9 MPa. Cell division appears less sensitive to water deficits (Hsiao et al., 1976). If stress is prolonged, however, cell division sensitivity in some cases can become as critical as is enlargement (Hsiao, 1973).

By reducing turgor, water stress can directly reduce stomatal opening and transpiration. Many studies have demonstrated this relationship and have indicated a threshold level of Ψ_w above which stomatal resistance remained constant. This critical level varies among species, but once exceeded, stomatal resistance usually increases rapidly with each small increase in water stress. For example, the threshold values are – 0.9 to -1.7 MPa for tomato, soybean, and grape (Hsiao, 1973); -1.5 to -2.5 MPa for peach and apple (Lankes, 1985).

The onset of photosynthesis inhibition is at about the same threshold value of Ψ_w as the stomatal resistance or lower Ψ_w level (Lakso, 1985). Sharkey and Seemann (1989) stated that stomatal closure was the main factor in inhibiting photosynthetic rates, but Harrison et al. (1989) suggested that the photosynthetic rate was directly influenced by water stress. Ward and Lawlor (1990) reported that photosynthesis could be inhibited by both stomatal and non-stomatal (biochemical) factors, such as inhibition of photochemical activity.

2.5.3 Osmotic adjustment

For physiologically active tissues: $\Psi_w = \Psi_s + \Psi_p$. When water stress develops, Ψ_w is lowered. To maintain adequate Ψ_p, the other component of tissue water potential, Ψ_s, must be lowered accordingly. The more negative Ψ_w is, the more negative Ψ_p must become.

In higher plants osmotic adjustment or osmoregulation refers to the lowering of Ψ_s. That arises from the net accumulation of solutes in response to water deficits or salinity (Turner and Jones, 1980; Morgan, 1984). The degree of osmotic adjustment is measured as the change in Ψ_s at a particular water potential or water content. The degree of osmotic adjustment commonly is measured at full turgor (i.e., when RWC = 100%, $\Psi_w = 0$, or $\Psi_s = \Psi_p$) (Turner and Jones, 1980) so that observed Ψ_s is converted to the corresponding value of Ψ_s at 100% RWC (Wang et al., 1995).

Changes in water content are passive responses to fluctuations in the solute concentration (Munns, 1988), whereas changes in the solute content resulting from metabolic activity are active responses (Bernstein, 1961). Passive osmotic adjustment by dehydration can account for 50 to 100% of Ψ_s changes, depending on species (Evans et al., 1992; Girma and Krieg, 1992) and the elasticity of cell walls. Active osmotic adjustment through net solute accumulation will maintain turgor and cell volume, as well as any turgor-mediated process (Hsiao et al., 1976). The solutes involved in active adjustment are sugars; glycerol; amino acids, such as proline or glycinebetaine; sugar alcohol such as sorbitol or mannitol; and other low molecular weight metabolites (Morgan, 1984; Voetberg and Sharp, 1991; Wang and Stutte, 1992).

Wang et al. (1995) found that osmotic adjustment occurred in leaves, stems, and roots of apple in response to water stress, and that adjustment was significantly higher in young leaves (about 1.0 MPa) than in mature ones (0.3 to 0.4 MPa). The amount of active osmotic adjustment in apple leaves could be up to 2.5 MPa over a season (Lakso et al., 1984). Water stress in apple leaves induced the accumulation of sorbitol while starch levels declined (Wang and Stutte, 1992).

Osmotic adjustment depends to a large extent on photosynthesis to supply compatible solutes (Morgan, 1984; Wang et al., 1995). As dehydration becomes severe, photosynthesis is inhibited and with a smaller solute supply, osmotic adjustment is curtailed. Thus, when water continues to be limiting, osmotic adjustment may delay but cannot completely prevent dehydration (Morgan, 1984; Munns, 1988).

2.5.4 Abscisic acid (ABA) development

ABA increases in response to water stress, subsequently inducing stomatal closure (Cornish and Zeevaart, 1985; Henson, 1985a; Davies and Zhang, 1991). ABA production requires gene transcription (Guerrero and Mullet, 1986). Mutants that cannot produce ABA are inferior in regulating their water relations (Karssen et al., 1990; Herde et al., 1997). During water shortages, ABA is synthesized in roots (Cornish and Zeevaart, 1985). It accumulates there (Robertson et al., 1985), where it affects water relations (Davies and Zhang, 1991), but it is also exported to the shoot (Zhang et al., 1987). The ABA level in stressed tissue readily returns to the pre-stress level after the plant is rewatered (Hsiao et al., 1976).

ABA affects stomatal aperture and also assimilate partitioning and growth (Henson, 1985b). Hartung et al. (1983) reported that ABA released into the apoplast during turgor or volume changes acted on photosynthesis and stomatal movement. Raschke (1982) speculated that stomatal closure at the leaf wilting point was probably mediated by ABA released into the apoplast. Pierce and Raschke (1980 and 1981) noted that in mesophyll cells of *Phaseolus vulgaris* ABA biosynthesis occurred at a threshold close to zero turgor in leaves.

2.6 Plant Adaptation to Water Stress

Adaptation is the modification in structure and function that increases the probability that the crop will survive and respond in a particular environment (Kramer and Boyer, 1995). Kramer (1980) classified plant drought adaptations as either drought avoidance (escape) or drought tolerance.

Drought avoidance is a characteristic of only a few plants, such as desert ephemerals and some plants growing in areas with well-defined wet and dry seasons. Drought-avoiding plants complete their life cycle, or at least their reproductive cycle, before the dry season begins. They are seldom severely stressed. Early maturation often is important when droughts occur late in the growing season (Kramer, 1980).

Drought tolerance can be subdivided into dehydration postponement and dehydration tolerance. Dehydration postponement is derived by morphological or physiological modifications that reduce transpiration or increase absorption. These may include tissue water storage, a heavily cuticulized epidermis that resists cuticular transpiration, efficient stomatal control of water loss, leaf abscission, extensive rooting

capacity (Pallardy, 1981), and the development of low Ψ_s. There are characteristics of many plant species formed in a more arid environment (Morgan, 1984).

Dehydration-tolerant plants endure protoplasmic loss of water or low water potentials without lethal injury. Although they are commonly lower plants such as mosses and bryophytes, certain higher plants can be desiccated by dry air without loss of viability (Bewley, 1979). The physiological basis for differences in dehydration tolerance among species may be at the molecular level, e.g., membrane structure and enzyme activity (Giles et al., 1976).

Most higher plants, including most woody plants, are either dehydration-postponing or drought-avoiding, rather than dehydration-tolerant (Levitt, 1980). However, Kramer (1980) emphasized that the these categories were not mutually exclusive because one kind of plant could have more than one type of adaptation. For example, some varieties of sorghum exhibit early maturity, and sorghum generally has extensive root systems and some degree of protoplasmic dehydration tolerance.

Stomatal closure in response to stress is a powerful mechanism for regulating water loss and reducing the development of further stress. Stomata are sensitive to stress when the leaf reaches a critical value of water potential; the stomata close rapidly after attaining the threshold level. This is an adaptive mechanism that enables the plant to photosynthesize briefly in the morning while the stomata are open and water status is above the threshold. This mechanism also keeps the plant in positive carbon balance and conserves water throughout the day (Beggs and Turner, 1976). Prompt stomatal closure in moderately stressed leaves may be advantageous in regions where drought is short-

term, but may be detrimental where drought lasts a long period, because stomatal closure cuts off CO_2 and reduces photosynthesis (Kramer and Boyer, 1995).

2.7 Methods of Reducing Water Loss of Plants

When transpiration exceeds water absorption under conditions of high evaporative demand, water stresses develop and growth is reduced. The amount of water transpired from a leaf may be significantly retarded by applying antitranspirants. The two main categories are film-forming and metabolic (stomatal-closing) antitranspirants.

2.7.1 Film-forming antitranspirants

Film-forming antitranspirants add additional resistance to the water loss pathway by partially covering or blocking stomata, thus reducing cuticular and stomatal transpiration (Davenport et al., 1972; Solarova et al., 1981). Permeability of the film to CO_2, which is often one-fourth that for water vapor, may cause a concurrent reduction in photosynthesis (Davis and Kozlowski, 1974).

Film-forming compounds are also effective in reducing transpiration in leafed-out deciduous trees (Gale and Hagan, 1966; Davies and Kozlowski, 1974; Ranney et al., 1989) and in evergreen conifers (Odlum and Colombo, 1987; Vera-Castillo, 1995). Englert (1992) reported that water loss from bare-root seedlings of *Quercus rubra*, *Acer platanoides*, and *Crataegus phaenopyrum* treated with antitranspirants, was reduced by up to 80% over untreated controls. Remmick (1995), however, reported the detrimental effects of root treatment with antitranspirants on the budbreak and establishment of dormant deciduous bare-root nursery stock such as dogwood (*Cornus florida*), river birch

(*Betula nigra*), Washington hawthorn (*Crataegus phaeopyrum*), and European beech (*Fagus sylvatica*).

Unfortunately, film-forming antitranspirants are often difficult to apply and do not obtain complete coverage. In general, the results are often disappointing (Englert, 1992).

2.7.2 Metabolic (stomatal-closing) antitranspirants

The natural plant growth regulator ABA is of interest because exogenously applied ABA lowers transpiration by closing stomata (Mansfield et al., 1978). Endogenous ABA levels increase as stomata close in droughted plants (Wright, 1977; Davies et al., 1980). Despite this, ABA may be unsuitable as a commercial antitranspirant because it is unstable in sunlight, expensive to synthesize, and is rapidly metabolized by plants (Davies and Mansfield, 1983; Hale and Orcutt, 1987).

Synthesized ABA-like compounds (structurally similar to ABA) may be potential antitranspirants. These ABA analogs are biotransformed into ABA in plants (Walton, 1983), and can be grouped into two classes. The first are compounds related to epoxy-β-ionylideneacetic acid. The second are acetylenic alcohols that act like ABA in germination and transpiration assays (Walton, 1983).

Farnesol and phenylmercuric acetate (PMA) are also endogenous antitranspirants. Mansfield et al. (1978) postulated that the function of farnesol in water-stressed sorghum was related to ABA distribution. Farnesol increased the chloroplast envelope membrane permeability to ABA, so that it could be transported from the mesophyll cells to the stomata. Farnesol probably triggered this ABA release.

With the exception of ABA (Jones and Mansfield, 1970), metabolic antitranspirants have generally proven toxic to a range of woody and herbaceous plants (Kozlowski and Clausen, 1970; Davenport et al., 1971).

2.7.3 Use of osmotic agents for stimulating water deficit stress in plants

Another way to reduce plant water loss is to induce stomatal closure by applying to the plant growth medium an effective amount of an osmotic agent. This can produce more negative soil water potential, thus stimulating water stress and inducing stomatal closure substantially earlier than under natural water stress conditions. This method and composition of the osmotic agent (GLK8924) were invented by the Great Lakes Chemical Corporation (West Lafayette, IN, USA). The active ingredient of the compound is sorbitol.

Compounds such as NaCl have long been used as osmotic agents in water stress studies (Bernstein, 1961; Joshi, 1976; Kawasaki et al., 1983). One of the hazards, however, is development of Na toxicity. Other osmotica include sucrose (Michel, 1972), mannose (Herold and Lewis, 1977; Herold, 1978), dextran (Johansson, 1970; Heyser and Naybors, 1981), mannitol (Bayley and Setterfield, 1957; Jackson, 1965), and sorbitol (Paleg and Aspinall, 1981; Bowman, 1988; Auge et al., 1992).

Sugar alcohols such as mannitol and sorbitol have often been used as metabolically inert osmotica (Paleg and Aspinall, 1981; Thompson et al., 1986; Bowman, 1988). Their structures (with six hydroxyl groups) are such that, in the absence of a specific transport system, they cross the plasmalemma very slowly, thus satisfying one suitability requirement (Cram, 1984). Bowman (1988) and Auge et al. (1992) reported

that lowering soil water potential with sorbitol solution significantly decreased stomatal conductance in *Andropogon glomeratus* and *Vigna unguiculata*, respectively

Attention during the past 35 years has been focused primarily on the use of PEG (polyethylene glycol) to simulate water stress effects in plants (Janes, 1966; Lawlor, 1970; Gergley et al., 1980; West et al., 1980; Yee and Rost, 1982; Robinson and Barritt, 1990). PEG is an inert, nonionic, long-chain polymer ($HOCH_2$-$(CH_2$-O-$CH_2)_X$ CH_2OH), and considered to be a nonpenetrating osmoticum (Applegate, 1960; Michel, 1971; Mexel et al., 1975; Oertli, 1985).

Kaufmann and Eckard (1971), Cress and Johnson (1987), and Hohl and Schopper (1991) concluded that PEG 6000 produced changes in plant water relations similar to those caused by drying soil at the same water potential. This was not the case, however, for PEG 400, which had toxic side effects (Kaufmann and Eckard, 1971). Other reports of undesirable side effects of PEG treatment have included reduction in oxygen content of the nutrient solution (Mexel et al., 1975) and visible leaf damage (West et al., 1980; Krizek, 1985).

Osmotic stress increases ABA concentrations in plants, as does water stress. Wolf et al. (1990) found that in *Lupinus albus*, salt treatment (40 $mol \cdot m^{-3}$) increased xylem ABA transport up to ten-fold and phloem transport to the root up to five-fold compared with controls. They concluded that, under adverse conditions, such as higher salinity, the root contributed more to ABA synthesis and transport than did the shoot. Robinson and Barritt (1990) showed that PEG 8000 (-1.0 MPa) stimulated about a ten-fold increase in free ABA concentrations in apical stem sections of apple one day after treatment.

2.8 Stomatal Control

2.8.1 Mechanisms of stomatal control

The most important role of stomata is to regulate gas exchange by controlling the turgor of guard cells and the aperture size (Mansfield, 1986). To open stomata requires an increase in guard cell turgor (Fisher and Hsiao, 1968; Mansfield, 1986). Early studies of stomatal control showed that changes in turgor were prompted by changes in the proportion of starch and sugar in guard cells (Lloyd, 1908; Sayre, 1926). When plants were exposed to light the photosynthetic process removed CO_2. The resultant increase in pH caused the hydrolysis of starch to sugar, therefore decreasing Ψ_s and increasing Ψ_p as water entered the guard cells. Fisher and his colleagues later reported that K^+ transport in and out of guard cells was chiefly responsible for changes in turgor (Fisher, 1968, Fisher and Hsiao, 1968; Fisher, 1971). The K^+ uptake was controlled by a plasmalemma-bound ATPase that pumped protons out of the cytoplasm (Fricker and Willmer, 1987). The concentration of K^+ in guard cells of open stomata was several times greater than that in the surrounding cells; correlation between the K^+ content of guard cells and stomatal aperture was significant (Fisher and Hsiao, 1968; Clint, 1985). When stomata closed, the apoplastic concentrations of K^+ increased. Blatt (1985) reported finding only a small pool of free K^+ in the apoplasts, while Bowling (1987) reported K^+ increased from 3 $mol \cdot m^{-3}$ to 100 $mol \cdot m^{-3}$ in the apoplasts during stomatal closure.

In the cell, K^+ is accompanied by its counter-anion (Cl^-) to balance the positive charge. Circadian rhythms of stomatal opening may be based on diurnal rhythms of K^+ and Cl^- transport out of or into the guard cells (Snaith and Mansfield, 1986).

The loss of K^+ that prompts stomatal closure can be induced by elevated levels of ABA around the guard cells (Mansfield and Jones, 1971; Ehret and Boyer, 1979; Fitzsimons and Weyers, 1987). This is probably the primary means for regulating stomatal closure (Harris and Outlaw, 1991). The ABA-induced loss of K^+ is related either to inhibited activity of proton-pumping ATPase (Schauf and Wilson, 1987) or to stimulation of the K^+ efflux (Fitzsimons and Weyers, 1987). Ehret and Boyer (1979) found a loss of K^+ in water-deficient leaves, regardless of the presence of roots. This suggested that local synthesis and metabolism of ABA probably accounted for much of the opening and closing response during water deficits.

Calcium ions can control the size of the stomatal aperture (Iljin, 1957). Adding calcium salts to the medium in which the isolated epidermis was incubated stimulated stomatal closure (and inhibited opening) in *Commelina communis* (Willmer and Mansfield, 1969; De silva et al., 1985a; McRobbie, 1989). The inwardly conducting K^+ channel was inhibited by mM concentrations of cytosolic calcium (Schroeder and Hagiwara, 1989; Schroeder and Hedrich, 1989).

Calcium ions acted synergistically with ABA by suppressing proton extrusion and ion-stimulated stomatal opening (Inoue and Katoh, 1987). De silva et al. (1985a and b) hypothesized that ABA increased the permeability of the guard cell plasma membranes for Ca^{2+} and operated as a secondary messenger to enable ABA to act on the ionic fluxes at the plasmalemma.

2.8.2 Factors affecting stomatal apertures

Environmental factors that influence stomatal opening and closing include light, CO_2 concentration, humidity, and temperature (Schulze, 1986). Internal factors include tissue water status and the level of plant growth regulators such as ABA and cytokinins. Their complex interactions often make it difficult to distinguish the relative importance of any single factor such as light and CO_2 or water status and ABA (Ball et al., 1987; Weyers and Meidner, 1990; Collatz et al., 1992).

2.8.2.1 Environmental factors

The blue light portion of the spectrum directly affects activation of proton extrusion by the guard cells, whereas red light acts via photosynthetic processes (Aphalo and Sanchez, 1986; Shimazaki and Zeiger, 1987). The blue light response may result from light absorption by flavin (Ogawa et al., 1978) while the response by red and part of the blue may be the result of light absorption by chlorophyll (Sharkey and Raschke, 1981). Both lights operate by affecting the amount and direction of ion transport across guard cell membranes (Serrano et al., 1988). Red- and blue-light-dependent stomatal opening can be reversed by far-red illumination, which demonstrates the involvement of the phytochrome system (Holmes and Klein, 1985; Holmes et al., 1986).

Schulze (1986) suggested that the ratio of the internal to the external CO_2 concentration is important in controlling stomatal aperture. The stomatal sensitivity to the external CO_2 concentration varies between C_3 and C_4 plants. Roger et al. (1983) reported that although the stomatal conductance (g_s) of corn, soybean, and sweet gum decreased about 50% when CO_2 concentration in open-topped outdoor chambers was

increased from 330 to 910 ppm, photosynthesis in soybean and sweet gum (both C_3 species) increased 65 to 70%. In contrast, there was no increase in photosynthesis by corn, which has a C_4 carbon pathway that is saturated at low CO_2 concentrations.

The reaction to CO_2 seems to vary with plant hormones such as IAA (indoleacetic acid) and ABA (Snaith and Mansfield, 1982; Raschke, 1986). Raschke and his group showed that if *Xanthium strumarium* plants were supplied with external ABA, or if production of endogenous ABA was stimulated by rapid transpiration or chilling (Raschke et al., 1976), then the stomata became sensitive to CO_2. Using isolated epidermis of *Commelina communis*, Snaith and Snaith and Mansfield (1982) showed that high concentrations of IAA in the incubation medium eliminated the inhibitory effect of CO_2 on the stomata, but this effect was counteracted by supplying ABA.

Stomatal movements in response to ambient humidity, without changes in bulk leaf Ψ_w, have been distinguished as feedforward responses from a feedback reaction (Nonami et al., 1990). The feedforward response is the phenomenon in which stomata respond to air humidity only because of a change in evaporative demand, but independently of the bulk water status of the leaf (Cowan, 1977; Farquhar, 1978). In contrast, a feedback regulation never causes reduced transpiration as evaporative demand increases, and a change in stomatal aperture occurs only after a change in bulk leaf water status (Cowan, 1977; Schulze, 1986). Evidence for two phases during stomatal movements was presented by Meidner (1986), who measured cuticular water losses under various humidity conditions, and by Kappen et al. (1987), who observed stomatal responses to humidity *in situ*. In response to increasing leaf to air vapor pressure

differences (VPD), a transient widening of the stomatal pores occurred first, followed by a stronger narrowing that continued under steady-state conditions. Aphalo and Jarvis (1991) found that g_s was better correlated with VPD than with humidity in *Hedera helix*; Assmann and Grantz (1990) had similar observations with sugarcane and sorghum.

2.8.2.2 Internal factors

Turner et al. (1985) showed that loss of turgor in leaf cells of *Helianthus annuus* was the principal cause of midday stomatal closure on dry, sunny days. However, in experiments on split root systems and the use of pressure to keep shoots turgid, when part of the root system is water deficient (Gollan et al., 1986; Davies and Zhang, 1991; Davies et al., 1993), signals (chiefly ABA) from drying roots caused stomatal closure, even in turgid shoots. Herde et al. (1997) showed that the absence of stomatal control over transpiration in ABA-deficient (*sitiens*) tomato could be restored if plants were then supplied with ABA. Stomata often failed to reopen immediately after water-deficient plants were rewatered and Fisher et al. (1970) attributed this to a persistently high concentration of ABA. Harris and Outlaw (1991), however, questioned this. In contrast, exogenous cytokinins increases stomatal aperture and transpiration rate and reversed closure caused by partial root drying or by exogenous ABA (Blackman and Davies, 1985; Incoll and Dewar, 1987).

2.8.3 Hydraulic control of stomatal conductance

The mechanisms of stomatal control underlying drought response are not well understood and have been vigorously debated (Sinclair and Ludlow, 1985; Kramer, 1988; Passioura, 1988; Boyer, 1989). The focus has largely been three issues: 1) the

relationship of leaf turgor to g_s; 2) the relative importance of hydraulic versus chemical effects of water stress on g_s; and 3) the role of roots, as opposed to shoots, as primary sensors of water stress.

There is no doubt that plants in drying soil show a decrease in bulk leaf Ψ_w and Ψ_p with a consequent reduction in g_s. Turner et al. (1985) demonstrated that the sudden onset of stomatal closure induced by water deficit was brought about by loss of turgor. However, in the long term, that stomatal closure may not have been correlated with the turgidity of leaves, because of the stomatal closure reaction, which limited water loss, and because of osmotic adjustment (Turner et al., 1985; Nonami and Boyer, 1990).

Kramer (1988) illustrated the uncertainty of the chemical effect of water stress on g_s especially in the field, in two ways. First, significant time was required for leaf water stress to be signaled to the roots and for hormones to be produced in the roots and transported to the shoots, because water flow in tree xylem generally lags 2-3 hr behind transpiration (Schulze et al., 1985). Second, in the field, shoots were usually water-stressed before roots were. This point contradicted suggestions by Zhang et al. (1987) that reduction in root tip turgor affected the synthesis of ABA and cytokinins.

Stomatal conductance may also depend on water potential gradients within the plant. The gradient in Ψ_w between roots and leaves is linearly dependent on the flux of water through the plant (Ritcher, 1973). Regulation of the flux by changes in g_s maintains leaf Ψ_w within set limits (Sperry et al., 1993). Meinzer and Grantz (1990) showed that stomatal and hydraulic conductances in sugarcane (*Saccharum* spp. hybrid) were coordinated as a plant developed, so that leaf Ψ_w remained constant over a range of

plant sizes and environmental conditions. Whitehead et al. (1996) suggested that hydraulic signals triggered by sudden changes in transpiration provided rapid means of communication within the plant, because the velocity of the pressure wave can approach the speed of sound in water (Malone, 1993). Malone (1993), Tardieu and Davies (1993), and Whitehead et al. (1996) proposed that the rapid transmission of a hydraulic change may activate the generation of a chemical signal in the leaves to regulate g_s.

2.8.4 Chemical control of stomatal conductance

Schulze (1986) reported that if roots were being stressed, stomata closed even when leaves were not experiencing water stress. Split-root experiments were most convincing (Gowing et al., 1990; Zhang and Davies, 1990b). The root system was halved; one part was provided with ample water (shoots remained turgid) while the other part was allowed to dry out. Stomata often closed in response to such an experimental system, suggesting some kind of signaling from the roots. Zhang and Davies (1990b) found close correlations among soil drying, decreased g_s, and ABA in the xylem sap; leaf ABA was not as closely correlated with g_s.

To demonstrate the existence of a direct signal from roots to stomata, Gollan et al. (1986) pressurized the whole root system to maintain full turgor in a leaf as the soil dried. They showed that when wheat and sunflower were subjected to progressive soil drying while the leaf Ψ_w was maintained near zero MPa, dry soil induced stomatal closure even though the leaves of these plants were always fully turgid. Schulze (1986) speculated that the root tip may have produced a signal which counteracted the effects of ABA and kept the stomata open.

Gowing et al. (1990) divided the roots of small apple trees into two containers. Drying the soil in one container restricted leaf expansion and leaf initiation, but with no obvious effect on shoot water relations. When roots in contact with the drying soil were severed, leaf growth rate and leaf conductance recovered to that of the well-watered controls. They concluded that shoot growth and leaf conductance were more likely restricted by the increased supply of an inhibitor (e.g., ABA), which originated in roots that were in contact with drying soil. Other split-root experiments by Blackman and Davies (1985) indicated that cytokinins may have been involved.

Reports have been made of a clear relationship between leaf conductance and soil water status and also of an apparent relationship between both of these variables and ABA concentration in xylem (Tardieu et al., 1992; Khalil and Grace, 1993). From field studies, Wartinger et al. (1990) with *Prunus dulcis* and Tardieu et al. (1992) with *Zea mays* showed an apparent effect of xylem ABA on leaf conductance, probably evidence for a central role by ABA in chemical signaling between roots and shoots and in the control of g_s in the field. In some species such as wheat and *Phaseolus vulgaris*, however, substantial antitranspirant activity in the xylem stream could not be attributed to ABA (Munns and King, 1988; Trejo and Davies, 1991).

A tight relationship between ABA concentration in xylem sap and g_s was presented by Wartinger et al. (1990) and Zhang and Davies (1989a and b, 1990b, and 1991) and they proposed that changes in xylem ABA concentrations in response to soil-drying may be a stress signal to regulate g_s. Tardieu and Davies (1993) suggested that transpirational flux of water may be significant in determining g_s by diluting the root-sourced signal. Recent studies addressed whether leaf conductance responded to ABA

flux (rather than ABA concentration) from the xylem (Schurr et al., 1992; Gowing et al., 1993; Jackson et al., 1995), but none confirmed that g_s was either determined by ABA flux or by the amount of ABA. Instead, g_s was related more to xylem ABA concentrations than to parameters such as ABA flux, leaf turgor, or leaf ABA concentrations (Gowing et al., 1993; Tardieu and Davies, 1993; Jackson et al., 1995).

The distribution of ABA through the plant is greatly influenced by the pH of the various plant compartments. Hartung et al. (1990) showed how soil-drying caused a redistribution of ABA because of the increasing pH of the xylem sap. They emphasized that this could explain rapid and substantial changes in shoot functioning.

The role of xylem ABA in controlling leaf conductance may lessen when soil-drying and plant water deficit become severe (Correia and Pereira, 1994). Wartinger et al. (1990) suggested the co-existence of negative signals (e.g., cytokinins) from soil drying with positive signals (e.g., ABA). Auge and Duan (1994) also noted interacting effects of ABA and cytokinins on growth and stomatal behavior of mycorrhizal plants.

The practical importance of stomatal control by chemical messages originating from dehydrating roots under field conditions was questioned by Kramer (1988). He maintained that, in the field, shoots were usually water-stressed before roots are. Therefore, it was unlikely that, in the field, roots were often the primary sensors of water stress or that biochemical signals from the roots were as important as the direct hydraulic effects of shoot water stress. This controversy was resolved, to some extent, in two substantial field investigations that demonstrated strong stomatal control by root-sourced ABA (Wartinger et al., 1990; Tardieu et al., 1992).

Yet, consistent evidence of correlations between stomatal behavior and leaf water status is difficult to overlook. Tardieu and Davies (1992) reported that stomatal sensitivity to the ABA message was modified and increased as leaf Ψ_w fell. Tardieu and Davies (1993) emphasized that a concept was inadequate for stomatal control based only on chemical signals (e.g. ABA from roots), as well as control by water relations alone. Describing an integration of hydraulic and chemical signals that controlled g_s of plants in drying soil, they concluded that the root message provided a means for the plants to sense the conditions of water extraction (soil water status and resistance to water flux) on a daily time scale. The short-term plant response to this message depended on evaporative demand.

2.9 Abscisic Acid Immunoassays

Immunoassays utilizing monoclonal antibodies to ABA provided rapid and sensitive methods for ABA measurements in minute amounts of plant material. Such methods can measure changes in ABA concentrations during development and in response to environmental stresses. ABA can be quantified by ELISA (enzyme-linked immunosorbent assay) (Walker-Simmons, 1987; Morris et al., 1988), RIA (radioimmunoassay) (Quarrie et al., 1988; Vernieri et al., 1989; MacKay et al., 1990). Very low concentrations of ABA can be detected by ELISA, e.g., 3×10^{-14} mol (Vernieri et al., 1989), and by RIA, e.g., 2×10^{-16} mol (Harris and Outlaw, 1991).

Immunoassay methods must be modified and verified for each type of plant tissue. With monoclonal antibodies, ABA can often be measured directly in crude extracts (Walker-Simmons, 1987) and after simple clean-up using C-18 cartridges

(MacKay et al., 1990). It is often preferable to extract ABA with water (especially boiling water) so that organic solvents do not interfere with the immunoassay (Quarrie et al., 1988).

ABA recovery controls should be conducted for each new plant tissue assayed to test for possible non-specific interference from substances in the plant tissue or the extraction medium. After a range of authentic ABA standards is added to the plant sample dilutions, the proportional increase of ABA measured by immunoassay is checked against the amount of ABA added (Jones, 1987). The accuracy of ABA measurements made with monoclonal antibodies compared with physico-chemical methods has been verified for ELISA (Norman et al., 1988) and RIA (Rosher et al., 1985; Quarrie et al., 1988).

CHAPTER 3

INFLUENCE OF SORBITOL-INDUCED OSMOTIC STRESS ON WATER RELATIONS AND STOMATAL CONDUCTANCE OF CONTAINER-GROWN APPLE (*Malus domestica* Borkh.) PLANTS

3.1 Abstract

To study the effect of sorbitol-induced osmotic stress (SIOS) on water loss reduction during water stress period, either water or sorbitol solution (-1.2 MPa) was applied to the plant growth medium of bench-grafted, potted 'Gala'/M26 apple (*Malus domestica* Borkh.) plants at a rate of 400 ml per 3.8-liter pot in a greenhouse. Thereafter water was withheld for 8 days and then rewatered. SIOS induced a rapid decrease in stomatal conductance in fully expanded leaves one hr after treatment. This was related to a rapid decrease in turgor potential from about 1.15 to 0.45 MPa, and was caused mainly by a substantial decrease in total leaf water potential (from -1.3 to -2.2 MPa). Osmotic potential was not affected. The relationship between leaf water status and stomatal conductance showed threshold values of -2.0 MPa water potential and 80% relative water content.

Because of the earlier reduction in stomatal conductance, SIOS enabled the plants to maintain a better water status than the water-stressed controls during the 8-day drying period. Afterward, leaf water potential and relative water content in the SIOS-treated plants were -3.5 MPa and 72%, respectively, compared to -4.2 MPa and 64% in the water-stressed controls.

During eight days of rehydration, stomatal conductance in the SIOS-treated plants was never restored because of the slower recovery in leaf water status (water potential and relative water content). Water-stressed controls recovered gradually to about 60% of the conductance of well-watered controls.

3.2 Introduction

Water deficit can cause serious losses in most crops, including apples. Water stress can affect plant processes such as cell growth (Acevedo et al., 1971), ABA synthesis (Cornish and Zeevaart, 1984), stomatal movement (Henson et al., 1989), CO_2 assimilation (Robinson et al., 1988), and osmotic adjustment (Wang et al., 1995).

Many plants develop either morphological and/or physiological features that enable them to resist water stress. Exogenous means have also been developed to improve water stress resistance. In one method, the stomata are physically plugged with surface-applied large organic polymers, i.e., film-forming antitranspirants (Murakami et al., 1990; Englert, 1992; Remmick, 1995). These antitranspirants can be difficult to apply for complete coverage and frequently are toxic to the leaf tissue. Results often depend on plant and environmental conditions (Englert, 1992). In a second method, water loss can be reduced by introducing compounds such as abscisic acid (ABA) into the plant, physiologically causing stomatal closure. ABA has inhibited transpiration in a wide range of species. However, it is expensive to synthesize, and is rapidly inactivated by sunlight and broken down by plants (Davies and Mansfield, 1983). Often, its effects are also temporary and/or inconsistent.

A water loss-reducing agent is needed, which is non-toxic to plants, environmentally acceptable, and relatively inexpensive. Recently, Great Lakes Chemical Corporation (West Lafayette, IN) patented an osmotic agent 'GLK8924' whose main ingredient is sorbitol. Besides reducing water loss, this compound was shown to effectively condition plants for improving water stress resistance. Unfortunately, the ability of this compound to reduce water loss was not physiologically evaluated.

Our study examined the effects of sorbitol-induced osmotic stress (SIOS) on water relations and stomatal conductance in apple. We tested the hypothesis that drenching with a hypertonic solution of sorbitol could increase desiccation resistance by reducing transpirational water loss. Stomatal closure would be induced earlier, compared to water-stressed controls. Bench-grafted, potted trees were chosen because of the economic importance of apples in Northwest nurseries. In addition, apple leaf tissue is convenient for determining water relations and gas exchange with a pressure chamber and porometer.

3.3 Materials and Methods

3.3.1 Plant material and stress imposition

Bench-grafted 'Gala'/M26 apple plants were potted in 3.8-liter plastic pots containing a 1:1:1:1 (by volume) mix of pumice, soil, coarse sand, and peat. They were grown in a lathhouse with 20% shade in the Department of Horticulture at Oregon State University, Corvallis. Osmocote (Sierra Co.), a slow-release fertilizer (20-20-20 plus micronutrients; five grams per pot), was incorporated. Plants were trained to one stem after axillary shoots were removed.

Three weeks before the experiment in August 1993, 135 trees were selected for uniformity and transferred to the greenhouse under day/night temperatures of 28°/18°C (± 2°C), natural light and photoperiod. Each tree was limited to 20 leaves. The trees were arranged on a bench in a randomized complete block design with 15 single-plant replicates and blocked according to light distribution. Factors were replication (block) and sampling time.

The potting medium was brought to field capacity before treatments began. Well-watered control plants were watered to field capacity daily throughout the experiment. For other plants, either water or sorbitol solution (-1.2 MPa) was applied as a soil drench at a rate of 400 ml per pot on Day 0 (at 12:30 p.m.), and water was then withheld for 8 days, for the respective water-stressed controls and sorbitol-treated plants. To determine the final concentration of this solution, one ml of leachate was collected 30 min later and its osmotic potential measured with a Wescor Vapor Pressure Depression Osmometer (model 5100 C, Wescor Inc., Logan, UT). The osmotic potential of sorbitol leachate was about -0.81 MPa (n=5). On Day 8, all water-stressed control and SIOS-treated plants were rewatered to complete saturation by keeping them in water-filled saucers for 30 min, and thereafter well-watered for another 8 days at 2- and 4-day intervals, respectively.

Water relations and stomatal conductance were assessed at 1- to 3-day intervals on both 'expanding' (upper 4 to 6) and 'fully expanded' (upper 12 to 14) leaves. Three replicates of each treatment were measured at each sampling time. On Day 0, a diurnal time course was determined at 1- to 2-hr intervals from 7:00 a.m. to 5:30 p.m.

3.3.2 Environmental conditions

Temperature, humidity, and photosynthetically active radiation (PAR) inside the greenhouse were monitored with a thermocouple and humidity and PAR sensors built in to the Li-Cor 1600 porometer chamber.

3.3.3 Volumetric soil water content

Volumetric soil water content was determined by time domain reflectometry (TDR), as described by Kelly et al. (1995). TDR probes were inserted in a pot at two horizontally equidistant positions, and readings were taken at 1- to 3- day intervals. Soil water content was determined from the mean of the two readings.

3.3.4 Stomatal conductance

Stomatal conductance, g_s, was measured between 11:00 a.m. and noon by using a Li-Cor 1600 steady state porometer with a manually controlled null-balance system (LI-COR, Inc., Lincoln, NE). The air flow rate was adjusted so that the relative humidity in the chamber did not change during each measurement period. Immediately after g_s was measured, the leaf was excised and kept in a plastic bag for later analysis of leaf water potential (Ψ_w), osmotic potential (Ψ_s), and relative water content (RWC).

3.3.5 Total leaf water potential and relative water content

Total leaf water potential was determined with a pressure chamber (PMS Instruments, Corvallis, OR), as described by Scholander et al. (1965). Briefly, a leaf was mounted inside a pressure chamber with the cut end of its petiole protruding outward through an airtight seal. Pressure in the chamber was gradually increased with

pressurized nitrogen until free water appeared at the cut surface. The reading at the incipient point of extruding water was taken as Ψ_w.

Relative water content was measured on a 1 cm leaf disk cut with a cork borer from the same leaf used for determining Ψ_w, and was calculated as

$$RWC = (Fwt - Dwt)/(Twt - Dwt) \times 100$$

where Fwt was the fresh weight immediately after the leaf disk was made, Twt was the turgid weight measured after the disk floated for three hr in ddH_2O, and disk Dwt was the dry weight after oven-drying at 80°C for two days.

3.3.6 Osmotic potential and osmotic adjustment

One half of the leaf (midribs excluded) that had been used for measuring Ψ_w was enclosed each in a 1.5 ml eppendorf tube immediately after Ψ_w measurement, and then frozen in liquid nitrogen and stored at -80°C. To determine Ψ_s, each frozen sample was smashed thoroughly with a glass rod for 30 s in the tube. A few drops of each exudate were decanted into another eppendorf tube and centrifuged at 12,000 g for five min to obtain a clear exudate. The osmolality of the supernatant was determined with a Wescor Vapor Pressure Depression Osmometer as described by Pomper and Breen (1996). Osmolalities were converted to MPa Ψ_s by multiplying by 2.48 (This was according to Van't Hoff's equation — $\Psi_s = -RT\Sigma C_j$, where R is the gas constant, T was the temperature in degrees Kelvin and C_j was the summation of the concentrations of all solutes in the solution). Cell sap Ψ_s was represented without correcting for dilution by apoplast solution. Turgor pressure was estimated as $\Psi_p = \Psi_w - \Psi_s$.

As is typically done, the degree of osmotic adjustment was shown by the difference between actual Ψ_s (determined after stress) and Ψ_s adjusted to 100% RWC (Wang et al., 1995).

3.4 Results

3.4.1 Environmental conditions

The average value of midday (noon) PAR in the greenhouse was approximately 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, with a maximum of 400 and a minimum of 160 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 3-1).

3.4.2 Volumetric soil water content

Soil water content in both water-stressed control and SIOS-treated plants decreased gradually through 8-day drying period; the rate of decrease was faster in the water-stressed controls (Fig. 3-2). By Day 8 soil water content was about 0.05 and 0.08 $m^3 \cdot m^{-3}$ for water-stressed controls and SIOS-treated plants, respectively.

3.4.3 Stomatal conductance and water relations

SIOS treatment immediately reduced g_s, regardless of leaf type. Measured one hr after treatment, g_s decreased from about 0.55 to 0.23 $cm \cdot s^{-1}$ in fully expanded leaves, and from 0.82 to 0.24 $cm \cdot s^{-1}$ in expanding leaves (Fig. 3-3). In general, diurnally maximum values of g_s were about 25% higher in expanding leaves than in fully expanded leaves. In general, the level of g_s in SIOS-treated plants was below 0.2 $cm \cdot s^{-1}$ on Day 1 and slowly decreased to near zero by Day 8 (Fig. 3-4). The g_s in water-stressed controls did not decline to below 0.2 $cm \cdot s^{-1}$ until Day 4.

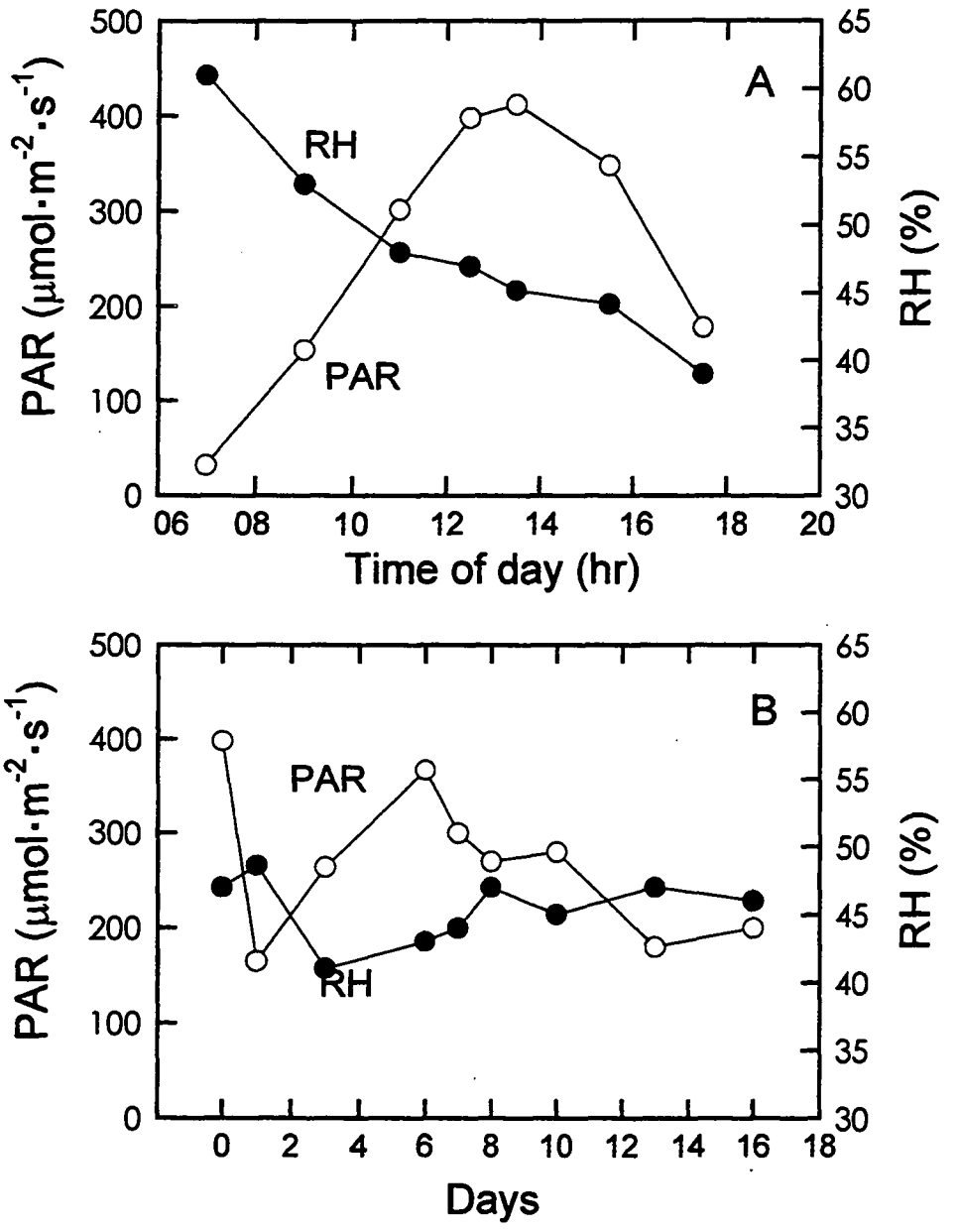

Figure 3-1. Changes in greenhouse photosynthetically active radiation (PAR) and relative humidity (RH) on Day 0 (A) and during eight days of water stress and after rewatering (B) in mid-September, 1994. Values are means ± SE (n=4). Day 0 in (B) is represented by midday reading.

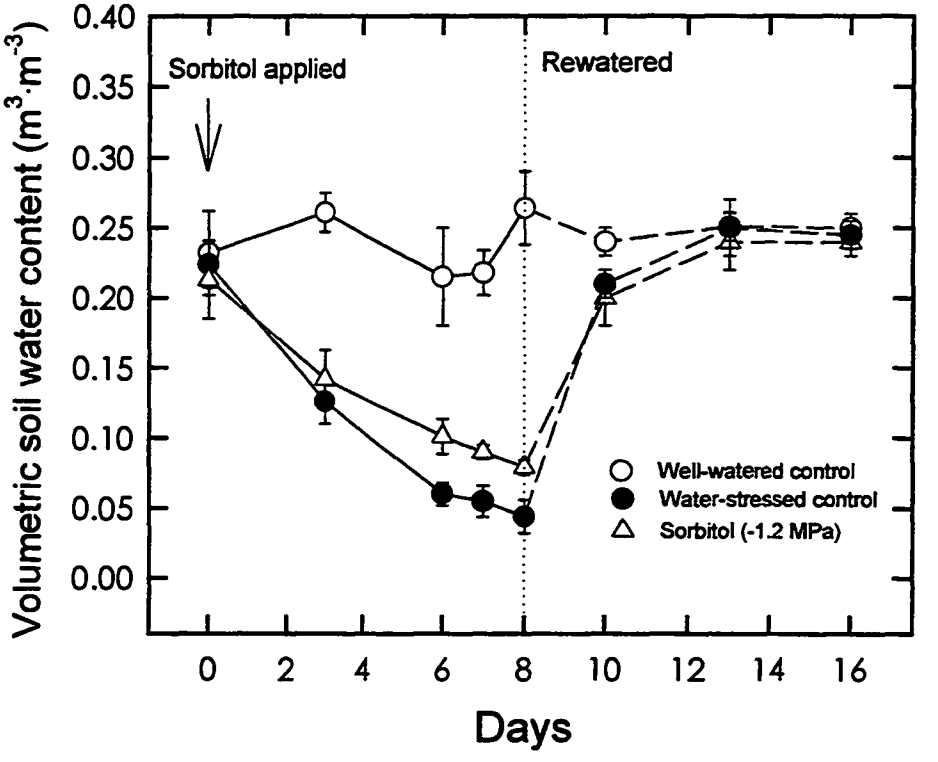

Figure 3-2. Changes in volumetric soil water content in media treated with either water or sorbitol (approximately -1.2 MP Ψ_s), with eight days of water stress and after rewatering. Water content was determined with time domain reflectometry (TDR).

Figure 3-3. Time course of stomatal conductance, g_s, and water relations of fully expanded (A) and expanding (B) apple (*Malus domestica* Borkh.) leaves of plants treated with either water or sorbitol (-1.2 MPa Ψ_s) as a soil drench during the first day of treatment. Ψ_w = total leaf water potential, Ψ_s = leaf osmotic potential, and Ψ_P = leaf turgor potential.

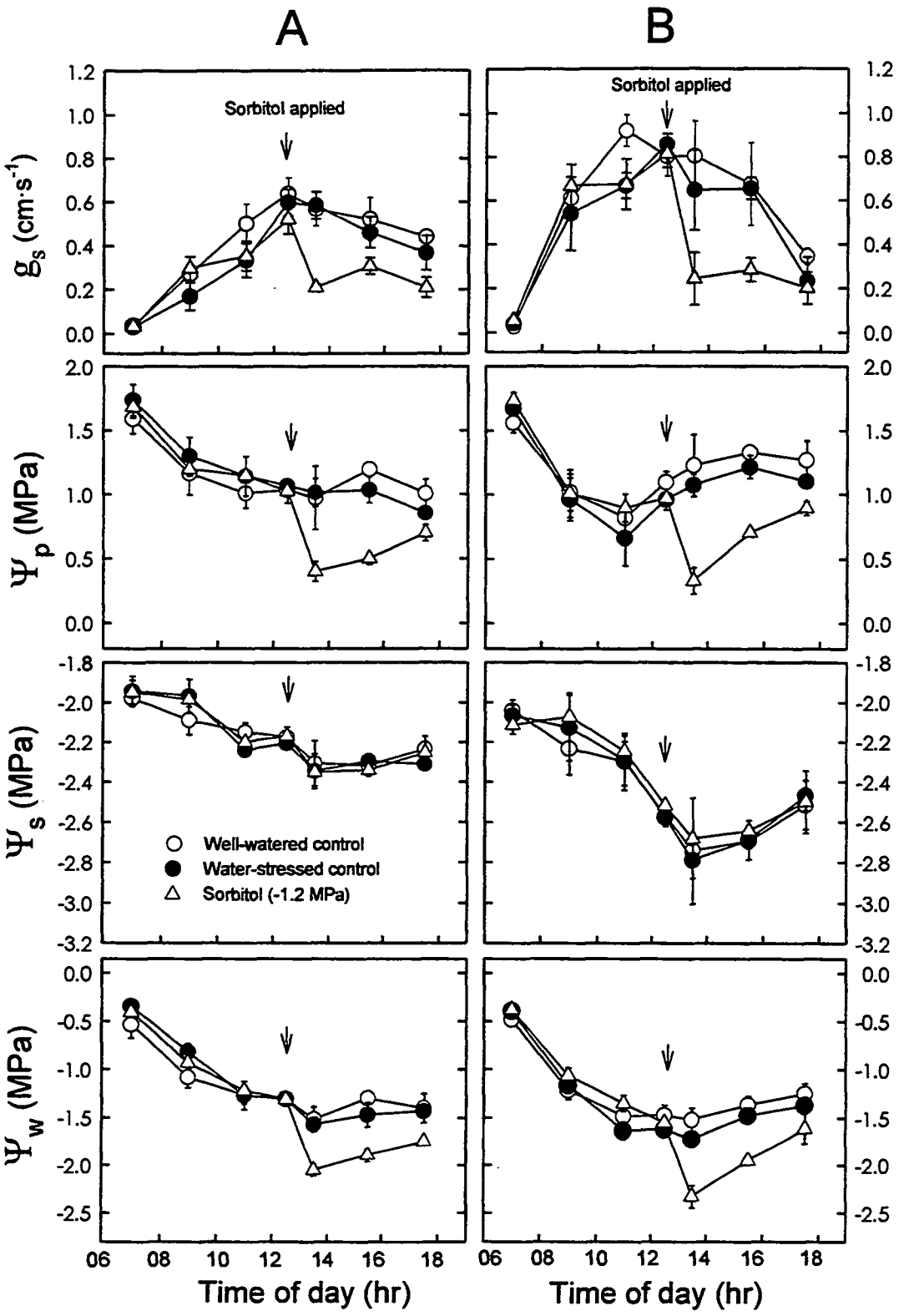
A
B
Sorbitol applied
Sorbitol applied
g_s (cm·s^{-1})
Ψ_p (MPa)
Ψ_s (MPa)
Ψ_w (MPa)
Well-watered control
Water-stressed control
Sorbitol (-1.2 MPa)
Time of day (hr)
Time of day (hr)

Figure 3-3

Figure 3-4. Time course of stomatal conductance, g_s, and water relations of fully expanded (A) and expanding (B) apple leaves of plants treated with either water or sorbitol (-1.2 MPa Ψ_s) as a soil drench during eight days of water stress and after rewatering. Ψ_w = total leaf water potential, Ψ_s = leaf osmotic potential, and Ψ_p = leaf turgor potential.

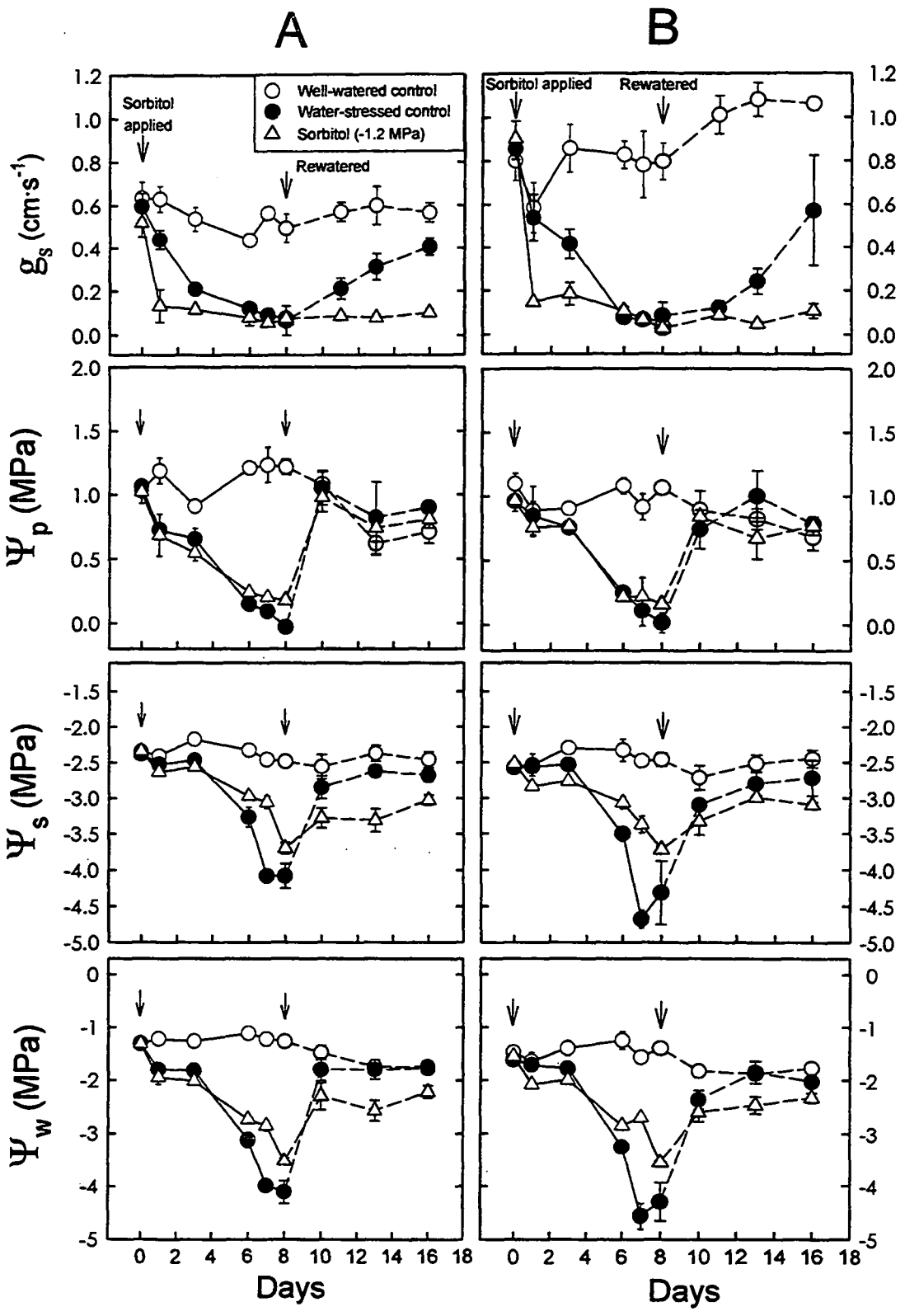
A
B
Well-watered control
Water-stressed control
Sorbitol (-1.2 MPa)
Sorbitol applied
Rewatered
g_s (cm·s^{-1})
Ψ_p (MPa)
Ψ_s (MPa)
Ψ_w (MPa)
Days

Figure 3-4

After rewatering, g_s in the water-stressed controls gradually recovered to 0.42 $cm \cdot s^{-1}$ (fully expanded leaves) and 0.58 $cm \cdot s^{-1}$ (expanding leaves) during the eight days after rewatering, but not to the level of well-watered controls; g_s in the SIOS-treated plants did not recover at all (Fig. 3-4).

In well-watered controls, Ψ_w ranged from -0.41 MPa early in the morning to -1.58 MPa at midday, regardless of leaf type (Fig. 3-3). Diurnal changes in Ψ_s and RWC, however, were greater in expanding leaves than in fully expanded leaves (Figs. 3-3 and 3-5). For example, Ψ_s for the former decreased from -2.12 to -2.83 MPa but only from -1.92 to -2.36 MPa for the latter.

SIOS treatment significantly reduced Ψ_w, Ψ_p, and RWC one hr after treatment, regardless of leaf type. For example, in fully expanded leaves, $\Delta\Psi_w$ was -0.77 MPa, $\Delta\Psi_p$ was -0.70 MPa (Fig. 3-3), and ΔRWC was 5% (Fig. 3-5). Toward late afternoon, Ψ_w and Ψ_p gradually recovered from their minimums to -1.73 MPa and 0.75 MPa, respectively, six hr after treatment. However, Ψ_s in the SIOS treatment was not affected, similar to the diurnal change in the well-watered controls.

After eight days of drying, water relation values were significantly lower in the water-stressed controls than in the SIOS-treated plants (Fig. 3-4). For example, in fully expanded leaves, Ψ_p in the water-stressed controls reached was below zero at the end of the drying period; Ψ_p in the SIOS-treated plants remained above zero. Similarly, the water-stressed controls reached -4.11 MPa Ψ_w and -4.08 MPa Ψ_s on Day 8; SIOS-treated plants were at -3.52 MPa Ψ_w and -3.4 MPa Ψ_s.

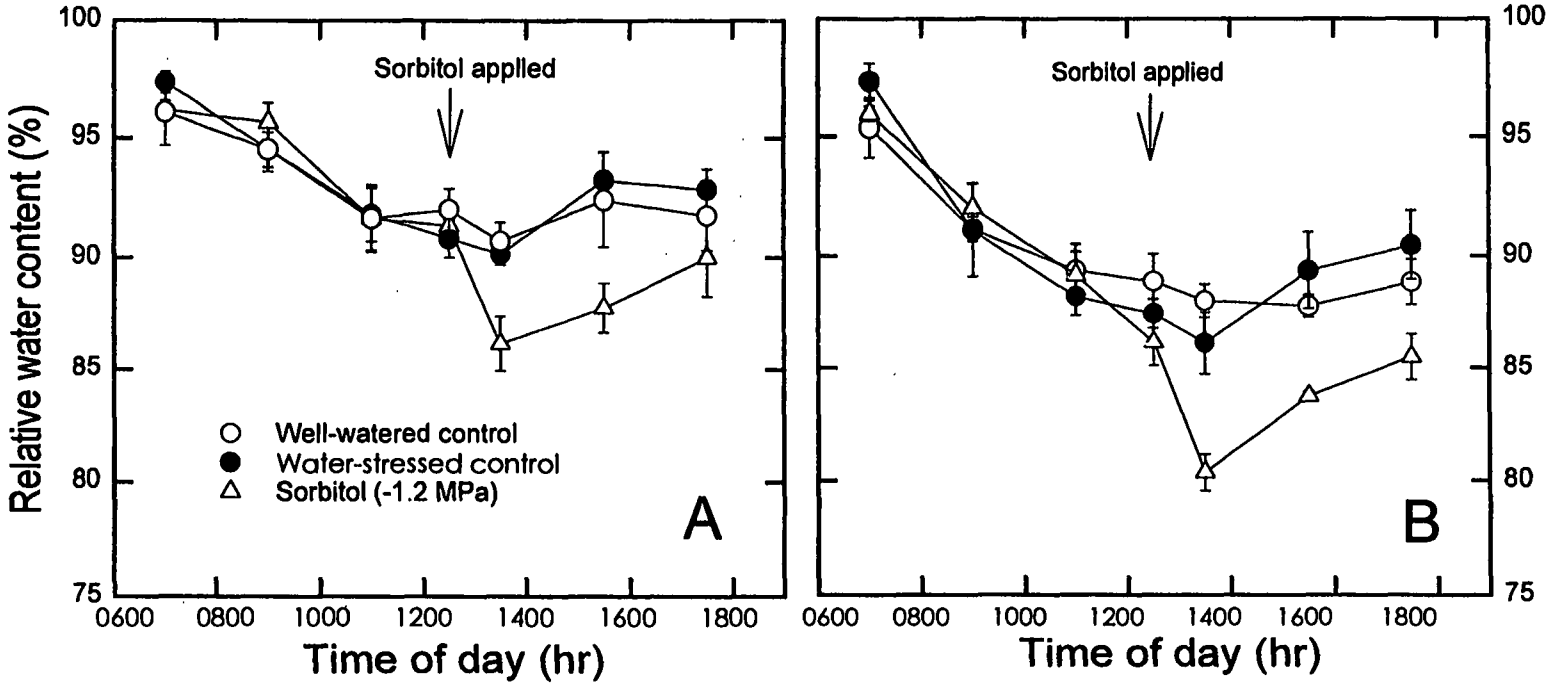

Figure 3-5. Time course of relative water content, RWC, of fully expanded (A) and expanding (B) apple leaves of plants treated with either water or sorbitol solution (-1.2 MPa) as a soil drench, during the first day of treatment.

After rewatering, recovery of Ψ_w, Ψ_s, and RWC from water stress was faster in the water-stressed controls than in the SIOS-treated plants (Figs. 3-4 and 3-6). However, after eight day rewatering, there was no difference in Ψ_p recovery between water-stressed control and SIOS-treated plants.

3.4.4 Osmotic adjustment

Diurnal measurements of osmotic adjustment showed that expanding leaves in well-watered controls were osmotically more adjusted (from -2.0 to -2.43 MPa Ψ_s at 100% RWC) than were fully expanded leaves (from -1.92 to -2.15 MPa Ψ_s at 100% RWC). SIOS-treated plants were less osmotically adjusted than were well-watered controls, as was clearly seen in expanding leaves (Fig. 3-7). With increasing water stress, however, SIOS-treated plants also showed osmotic adjustment, although the rate of adjustment seemed to be slower than in water-stressed controls (Fig. 3-8). After rewatering, adjusted Ψ_s gradually decreased, occurring faster in water-stressed controls than in SIOS-treated plants (Fig. 3-8).

3.4.5 Relationship between stomatal conductance and water relations

Stomatal conductance was positively correlated to all parameters of water relations regardless of treatment, except for Ψ_s in water-stressed controls (Figs. 3-9, 3-10, and 3-11). The threshold values of Ψ_w, Ψ_s, and RWC for inducing stomatal closure were about -2.0, -2.5 MPa, and 85%, respectively, regardless of leaf type (Figs. 3-9, 3-10, and

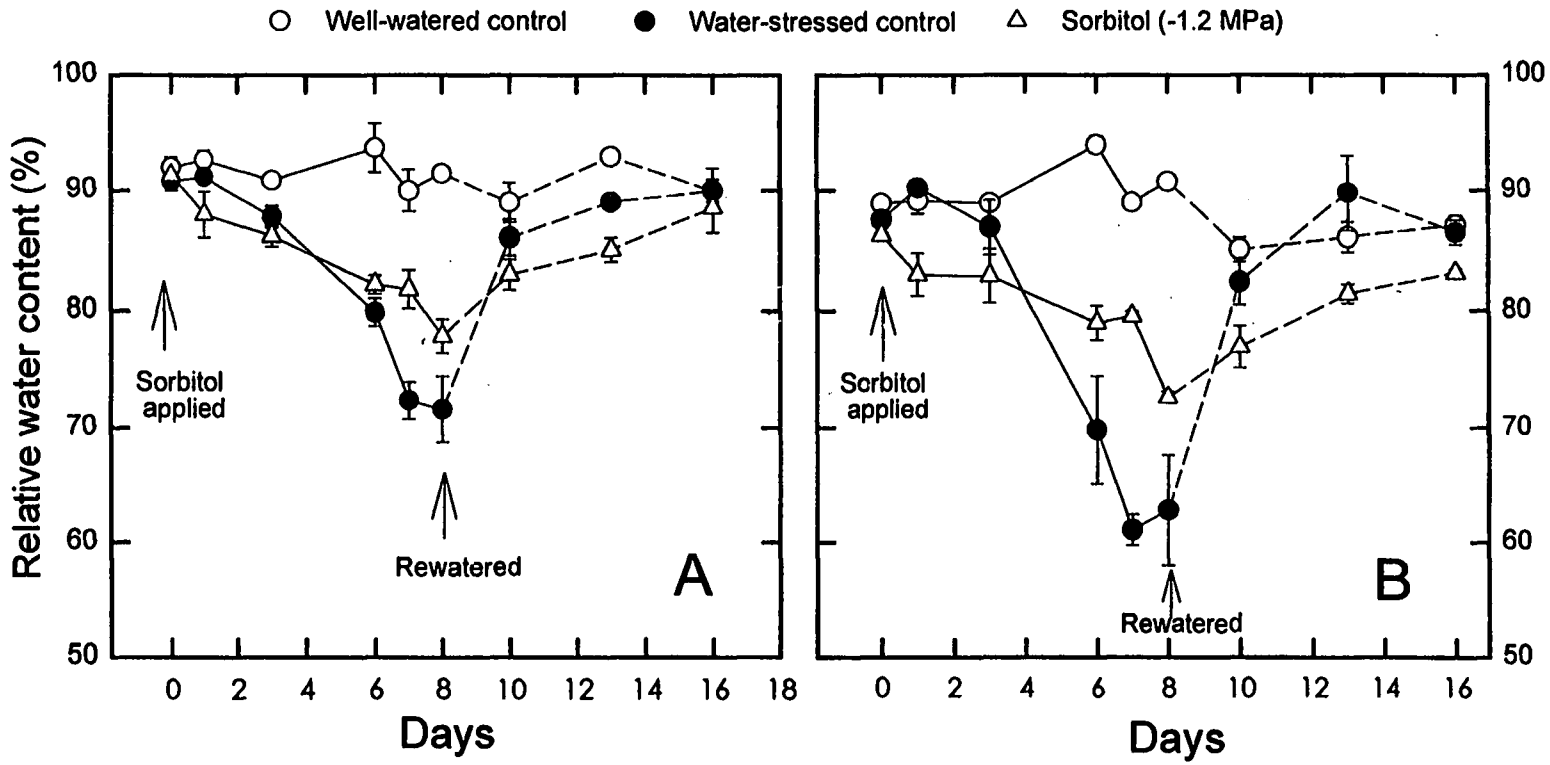

Figure 3-6. Time course of relative water content, RWC, of fully expanded (A) and expanding (B) apple leaves of plants treated with either water or sorbitol solution (-1.2 MPa) as a soil drench, during eight days of water stress and after rewatering.

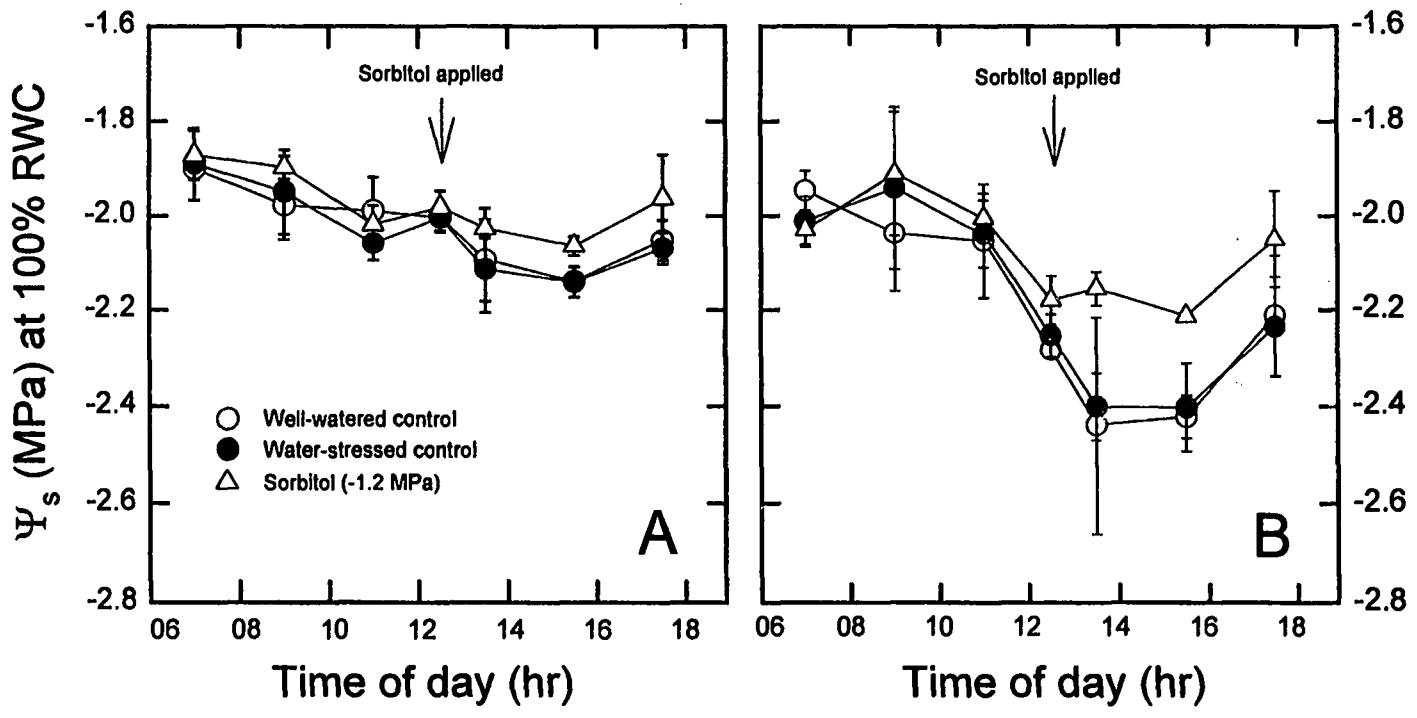

Figure 3-7. Changes in osmotic potential (Ψ_s) adjusted to 100% relative water content (RWC) of fully expanded (A) and expanding (B) apple leaves of plants treated with either water or sorbitol solution (-1.2 MPa) as a soil drench during the first day of treatment.

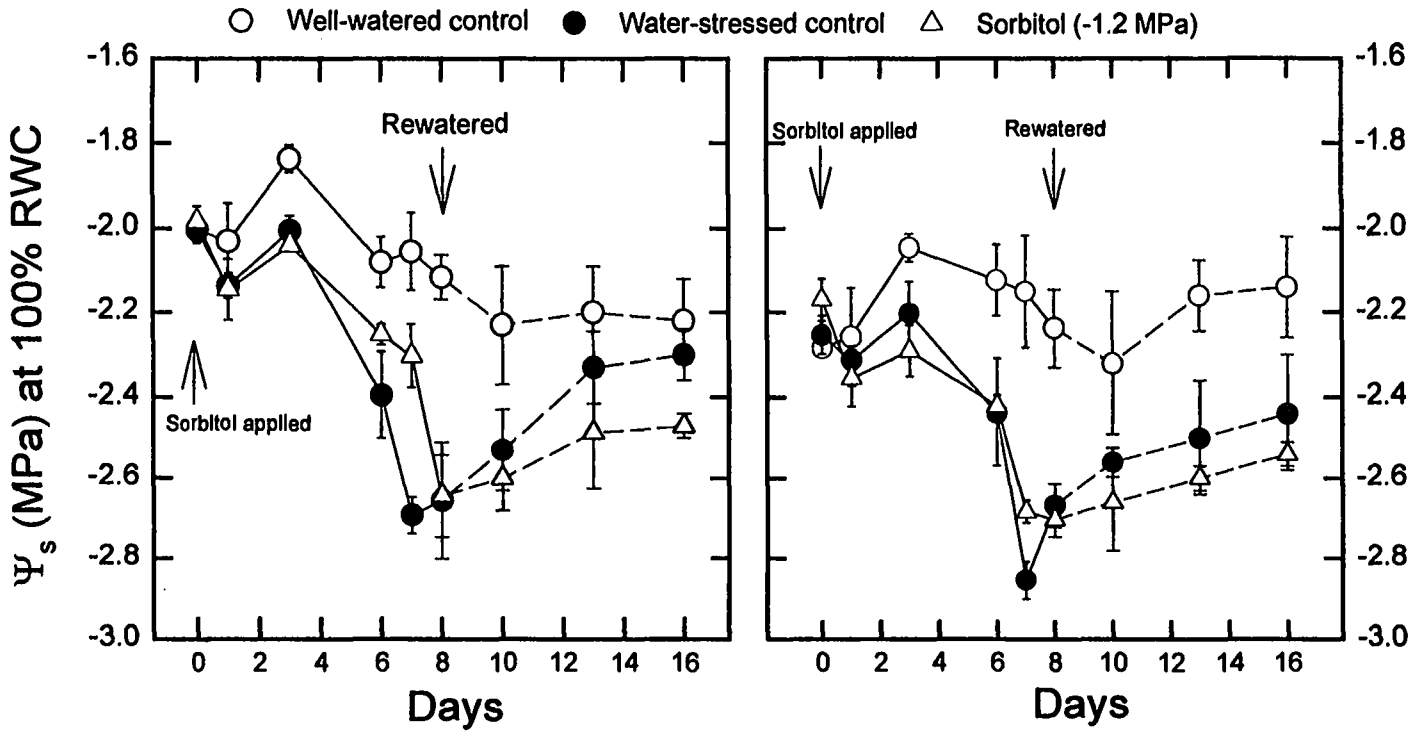

Figure 3-8. Changes in osmotic potential (Ψ_s) adjusted to 100% relative water content (RWC) of fully expanded (A) and expanding (B) apple leaves of plants treated with either water or sorbitol solution (-1.2 MPa) as a soil drench during eight days of water stress and after rewatering.

Figure 3-9. Relationship between stomatal conductance, g_s, and leaf (A) water potential, Ψ_w, (B) osmotic potential, Ψ_s, and (C) turgor potential, Ψ_P, of fully expanded apple leaves of plants treated with either water or sorbitol (-1.2 MPa) as a soil drench, during eight days of water stress. For the figures in insets, data collected through the first three days of drying period (to obtain values up to the threshold points) including time course (between 11:00 a.m. and 3:30 p.m.) of the first day of treatment were used. Regression equations for the figures in insets: (A) for water-stressed controls (solid line), $g_s = 1.161 + 0.046\Psi_w$ ($R^2 = 0.41$; $P = 0.0117$); for sorbitol-treated plants (short-dashed line), $g_s = 0.918 + 0.350\Psi_w$ ($R^2 = 0.49$; $P = 0.0006$), (B) for water-stressed controls leaf, $g_s = 0.584 + 0.051\Psi_s$ ($R^2 = 0.001$; $P = 0.486$); for sorbitol-treated plants, $g_s = 3.571 + 1.314\Psi_s$ ($R^2 = 0.84$; $P = 0.0001$), and (C) for water-stressed controls leaf, $g_s = -0.019 + 0.532\Psi_P$ ($R^2 = 0.48$; $P = 0.017$); for sorbitol-treated plants, $g_s = 0.074 + 0.295\Psi_P$ ($R^2 = 0.23$; $P = 0.021$).

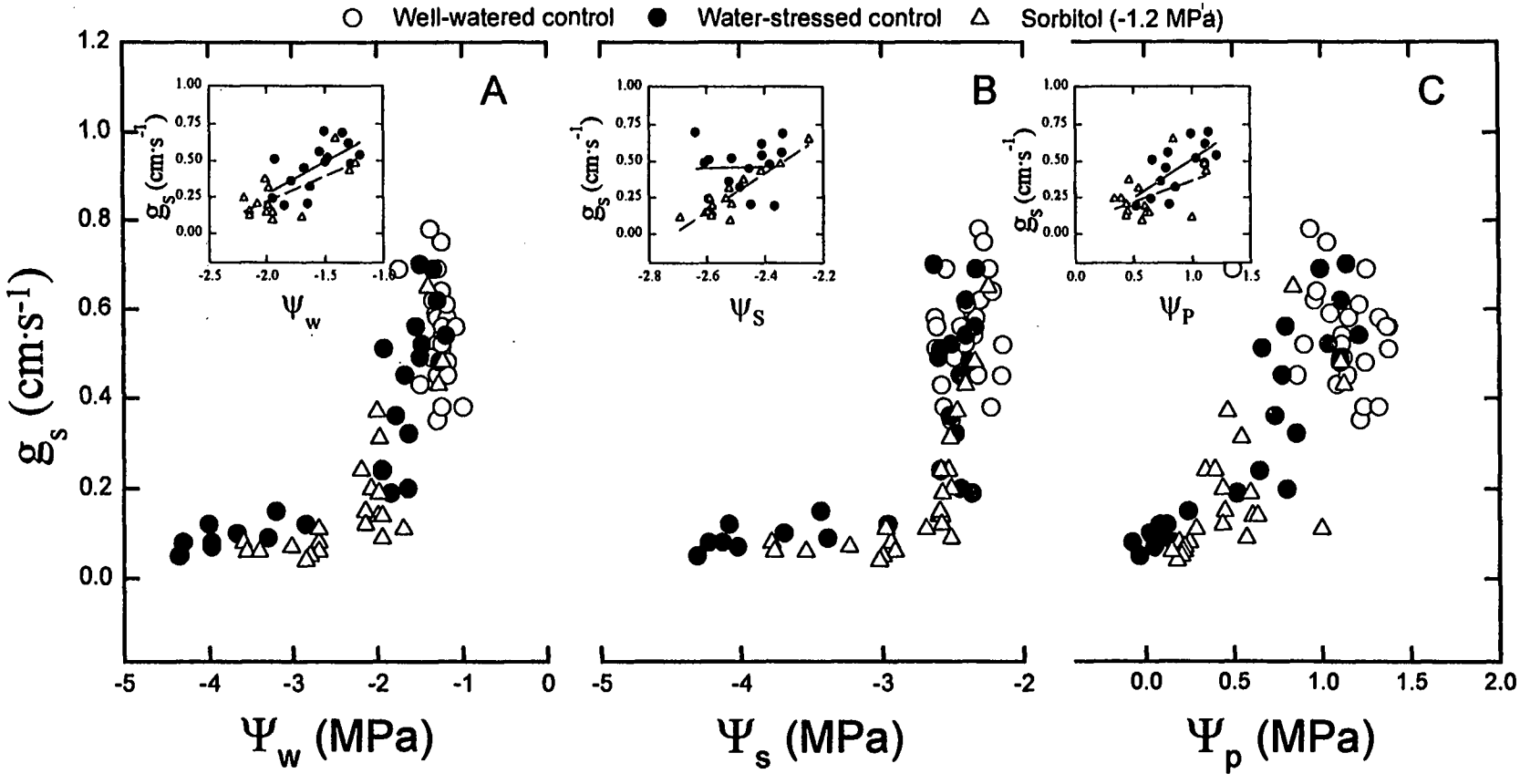
Well-watered control
Water-stressed control
Sorbitol (-1.2 MPa)
A
B
C
g_s (cm·s^{-1})
Ψ_w (MPa)
Ψ_s (MPa)
Ψ_p (MPa)

Figure 3-9

Figure 3-10. Relationship between stomatal conductance, g_s, and leaf (A) water potential, Ψ_w, (B) osmotic potential, Ψ_s, and (C) turgor potential, Ψ_P, of expanding apple leaves of plants treated with either water or sorbitol (-1.2 MPa Ψ_s) as a soil drench, during eight days of water stress. For the figures in insets, data were collected through the first three days of the drying period (to obtain values up to the threshold points) and included the time course between 11:00 a.m. and 3:30 p.m. on Day 0. Regression equations for the figures in insets: (A) for water-stressed controls (solid line), $g_s = 1.643 + 0.615\Psi_w$ ($R^2 = 0.15$; $P = 0.149$); for sorbitol-treated plants (short-dashed line), $g_s = 1.893 + 0.791\Psi_w$ ($R^2 = 0.62$; $P = 0.005$), (B) for water-stressed controls leaf, $g_s = -0.896 - 0.577\Psi_s$ ($R^2 = 0.13$; $P = 0.179$); for sorbitol-treated plants, $g_s = 5.298 + 1.846\Psi_s$ ($R^2 = 0.65$; $P = 0.003$), and (C) for water-stressed controls leaf, $g_s = 0.127 + 0.510\Psi_P$ ($R^2 = 0.25$; $P = 0.060$); for sorbitol-treated plants, $g_s = -0.175 + 0.708\Psi_P$ ($R^2 = 0.31$; $P = 0.032$).

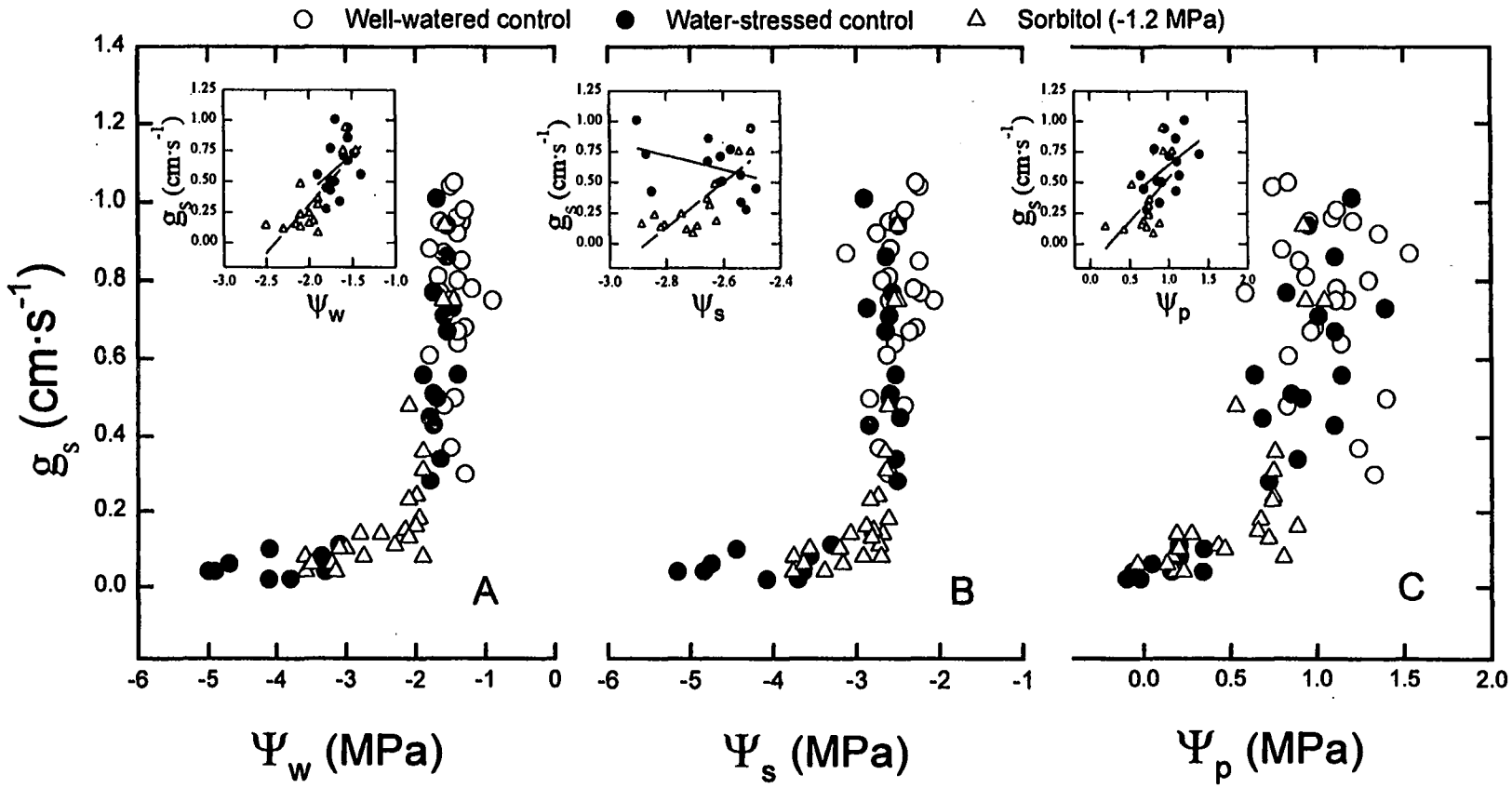
Well-watered control
Water-stressed control
Sorbitol (-1.2 MPa)
g_s (cm·s^{-1})
Ψ_w (MPa)
Ψ_s (MPa)
Ψ_p (MPa)
A
B
C

Figure 3-10

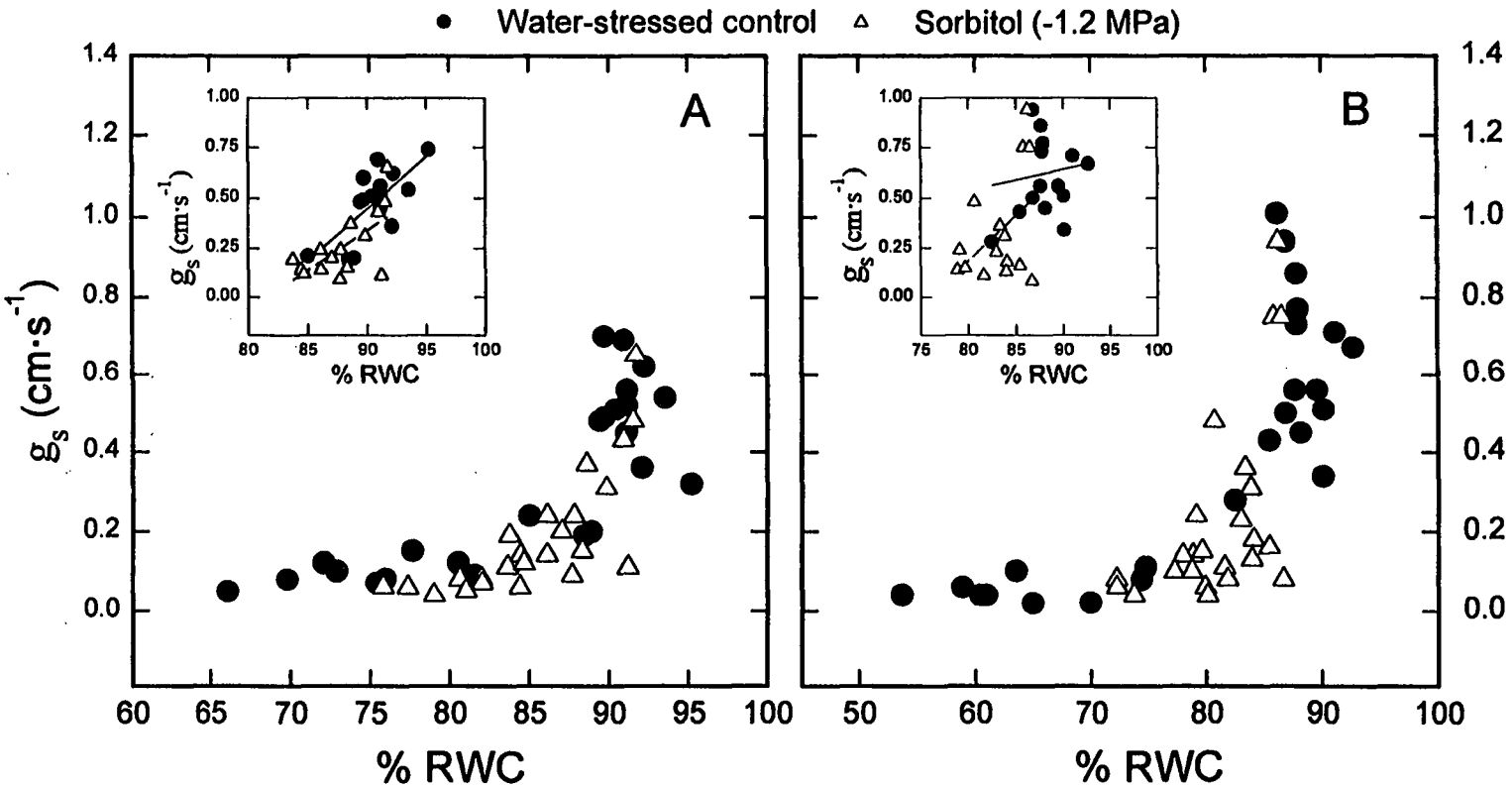

Figure 3-11. Relationship between stomatal conductance, g_s, and relative water content, RWC, of fully expanded (A) and expanding (B) apple leaves of plants treated with either water or sorbitol solution (-1.2 MPa) as a soil drench, during eight days of water stress. For the figures in insets, data were collected through the first three days of drying period (in order to obtain values up to the threshold points) and included the time course between 11:00 a.m. and 3:30 p.m. on Day 0. Regression equations for the figures in insets: (A) for water-stressed controls (solid line), $g_s = -0.311 + 0.01RWC$ ($R^2 = 0.015$; $P = 0.249$); for sorbitol-treated plants (short-dashed line), $g_s = -3.431 + 0.045RWC$ ($R^2 = 0.20$; $P = 0.045$) and (B) for water-stressed controls, $g_s = -4.231 + 0.052RWC$ ($R^2 = 0.51$; $P = 0.0012$); for sorbitol-treated plants, $g_s = -3.248 + 0.040RWC$ ($R^2 = 0.43$; $P = 0.0057$).

3-11). In contrast, the threshold value of Ψ_p was not as distinct, but g_s generally decreased linearly with Ψ_p

3.5 Discussion

Applying a hypertonic solution (-1.2 MPa) of sorbitol as a soil drench effectively reduced water loss during a short-term drying period compared with water-stressed controls. Water loss reduction in the plants treated with sorbitol-induced osmotic stress (SIOS) was caused by earlier stomatal closure (58-70% g_s of well-watered controls) than in water-stressed controls. The significant reduction in stomatal conductance (g_s) one hr after treatment was closely associated with the concurrent decline in leaf Ψ_w or Ψ_p. There was no correspondence with Ψ_s. So, based on $\Psi_p = \Psi_w - \Psi_s$, turgor decline caused by SIOS treatment was mainly attributed to the reduction in Ψ_w. Robinson and Barritt (1990) reported that PEG-induced water stress resulted in a rapid reduction in midday Ψ_p, paralleling the severity of the stress.

The role of leaf ABA in stomatal closure in response to SIOS-treated plants cannot be ruled out, because leaf ABA production usually depends on changes in leaf Ψ_p (Pierce and Raschke, 1981). Robinson and Barritt (1990) reported that ABA concentration of the mature apple leaf was negatively correlated to leaf turgor on Day 1 of the PEG (-1.0 MPa) treatment. Guak (1998; chapter 4) also showed that, under greenhouse conditions, ABA concentrations in mature apple leaves significantly increased two hr after SIOS treatment, which were closely related to decreases in g_s and Ψ_p by 72% and 67%, respectively. In contrast, when a similar experiment was conducted with plants grown outdoors, SIOS did not significantly increase leaf ABA concentrations,

but g_s and Ψ_p decreased by 55% and 25%, respectively. This difference seen between the two conditions suggests that a hydraulic signal generated by SIOS treatment (rather than a chemical signal such as ABA) may play a role in stomatal closure, especially early on. Saliendra et al. (1995) suggested that any treatment or event that effected a change in soil water potential or hydraulic conductance would result in hydraulic signals generated in the roots. This signal could be transmitted much faster than a chemical signal carried in the transpirational stream (Malone, 1993; Whitehead et al., 1996). It was also known that the rapid transmission of a hydraulic change triggers the generation of a chemical signal in the leaves to regulate g_s (Hartung et al., 1990; Malone, 1993; Tardieu and Davies, 1993).

Compared with those of SIOS-treated plants, g_s and Ψ_s were poorly correlated in water-stressed controls perhaps because the effects of osmotic adjustment on stomatal control varied by treatment. Compared with the SIOS treatment, control plants with relatively slow water stress imposition at least in the early drying period (before reaching the threshold Ψ_w) had g_s that did not readily decrease with decreasing Ψ_s because turgor was maintained by osmotic adjustment. In contrast, in the SIOS-treated plants subjected to relatively rapid water stress, g_s that was reduced immediately after treatment did not recover even after plants were osmotically adjusted to the level of water-stressed control (e.g., one day after treatment). Turgor became similar to that of water-stressed controls and g_s decreased with decreasing Ψ_s. Rapid water stress induced by SIOS could override the effects of osmotic adjustment on stomatal control. The physiological effects of

gradual water stress induced by withholding water may be quite different from those of rapid stress caused by applying an osmoticum (Krizek, 1985).

Osmotic adjustment did not occur in the SIOS-treated plants during the first day of treatment, compared to the well-watered controls. This may have been because the osmotic shock reduced ion uptake (Munns et al., 1979; Wyn Jones, 1979) and the production of photosynthates (Lakso, 1985; Wang et al., 1995). However, one day later, plants were osmotically adjusted to the level of water-stressed controls (Fig. 3-8), which seemed to be caused by sorbitol penetration into the plant. In addition, the osmotic shock-induced synthesis of secondary products like betaine (Wyn Jones and Storey, 1981; Zuniga et al., 1990) and proline (Premachandra et al., 1995), and/or the breakdown of carbohydrates into smaller units may have increased osmotic potentials (Lakso, 1985). Trip et al. (1969) reported that sorbitol or mannitol was taken up and metabolized rapidly in leaves in a wide range of species. Cram (1984), however, showed that sorbitol and mannitol penetrated relatively slowly across the plasmalemma and tonoplast of excised carrot storage root tissues, because they have six hydroxyl groups each.

After rewatering, regardless of leaf type, stomata of the SIOS-treated plants remained closed throughout the next 8-day rewatering period; stomata in the water-stressed controls gradually opened. In fully expanded and expanding leaves, Ψ_p in both treatments commonly recovered fully to the level of the well-watered controls two days after rewatering; Ψ_w and RWC recovered more slowly in the SIOS-treated plants. This contrast in the recovery of leaf water status (e.g., Ψ_w and RWC) might explain the difference in the extent of stomatal opening between the two treatments. Fisher et al.

(1970), however, assessed post-stress stomatal opening in leaf disks floating on water (to ensure full turgor) and clearly showed that the after-effect was not caused by the persistent water deficit. A slower reduction was possible in the level of an inhibitor such as ABA, on stomatal opening in SIOS-treated plants than in water-stressed controls (Mansfield and Jones, 1971; Slovik and Hartung, 1992). Guak (1998; chapter 4) showed, however, that the persistent stomatal closure even after SIOS-treated plants were rewatered was not related to the difference in the reduction of bulk leaf ABA after rewatering. Although leaf ABA concentrations in both treatments decreased to levels similar to those of well-watered controls, gradual increase in stomatal conductance occurred only in the water-stressed controls. The longer after-effect during recovery in the SIOS-treated plants may prevent excessive transpiration during periods of water shortage.

In conclusion, SIOS-induced water stress caused a rapid decrease in stomatal conductance in the greenhouse-adapted apples and induced significantly less desiccated-plants through the eight-day drying period compared with water-stressed controls. The reduction in stomatal conductance was closely associated with a substantial decrease in leaf turgor one hr after SIOS treatment as a soil drench. The after-effect of SIOS-induced water stress on stomatal opening persisted much longer than for water-stressed controls after rehydration.

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CHAPTER 4

EFEFCT OF SORBITOL-INDUCED OSMOTIC STRESS ON THE CONCENTRATION OF BULK LEAF ABSCISIC ACID IN CONTAINER-GROWN APPLE (*Malus domestica* Borkh.) PLANTS, AND ITS RELATIONSHIP TO STOMATAL CONDUCTANCE

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Effect of sorbitol-induced osmotic stress on the concentration of bulk leaf abscisic acid in container-grown apple (*Malus domestica* Borkh.) plants, and its relationship to stomatal conductance

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ADDITIONAL INDEX WORDS. ABA, osmotic potential, radioimmunoassay (RIA), turgor potential, stomatal after-effect, water relations, water potential.

4.1 Abstract

The effects of sorbitol-induced water stress (SIOS) on bulk leaf ABA concentrations and stomatal conductance (g_s) as well as leaf water relations were studied in bench-grafted 'Gala'/M26 apple plants (*Malus domestica* Borkh.) to test the hypothesis that application of sorbitol solution as a soil drench stimulates the production of leaf ABA which reduces g_s. Treatments included well-watered control, water-stressed control, and sorbitol solution (-1.2 MPa) application as a soil drench. After treatment, thereafter, no additional water was applied during 7 days of water stress for water-stressed control and SIOS-treated plants. Soil water content and leaf water status were correlated with leaf ABA production, which was highly correlated with g_s. Using radioimmunoassay of a monoclonal antibody, a rapid increase (up to 60% of the well-watered control) in leaf ABA of the SIOS-treated plants was detected two hr after treatment. This increase in bulk leaf [ABA] corresponded to a significant decline in the

leaf turgor and g_s. In the water-stressed control, however, leaf [ABA] increased to a level similar to that of SIOS-treated plants on day 3 when g_s decreased significantly. After plants were rewatered, leaf [ABA] for both treatments held steady or increased slightly for 2-4 days, then decreased to the well-watered control level. The after-effect of water stress on stomatal opening persisted, even after rewatering, and lasted much longer in the SIOS treatment than in the water-stressed control. Although the elevated ABA levels of the water-stressed control seemed to be related to the stomatal after-effect, this pattern in SIOS-treated plants was not similarly associated because stomatal opening never occurred even after bulk leaf [ABA] finally declined to the level of well-watered controls or water-stressed controls.

4.2 Introduction

Plants respond to water either by postponing dehydration while maintaining a high plant water potential (i.e., drought avoidance), or by tolerating dehydration with survival at low plant water potential (i.e., drought tolerance) (Kramer, 1983; Turner, 1986). Repeated nonlethal water stress (conditioning) has been used to improve tolerance to subsequent stress (Edwards and Dixon, 1995; Kramer, 1983; Zwiazek and Blake, 1988), although the physiological mechanisms involved are poorly understood (Buxton et al., 1985).

Water stress can cause a rapid accumulation of ABA in many plants, including apple (Robinson and Barritt, 1990; Walton, 1980; Wright and Hiron, 1969). This results from *de novo* synthesis rather than release from a bound form of ABA, as in red kidney bean (Pierce and Raschke, 1980) and Scotch pine (Hogue et al., 1983). Davies and Lakso

(1979) and Robinson and Barritt (1990) observed in mature apple leaves ABA accumulation as a response to water stress, and concluded that the increases in ABA are correlated with cell turgor pressure rather than water potential. The increases in ABA are presumed to cause stomata to close (Jones and Mansfield, 1970).

There is no doubt that plants in drying soil experience a decrease in bulk leaf water potential and leaf turgor with a consequent reduction in stomatal conductance and growth. However, roots in drying soil can affect shoot physiology independently of shoot water potential (Bates and Hall, 1981; Grantz, 1990; Livingston and Black, 1987; Saliendra et al., 1995). Over the last decade, analyses have served mainly to invalidate the theory of stomatal control by leaf water status alone and to demonstrate the controlling effect of chemical messages from roots to shoots (e.g. Davies and Zhang, 1991). Despite this, it is difficult to overlook consistent evidence, from the past 20 years, of a correlation between stomatal behavior and leaf water status. Tardieu and Davies (1992) and Tardieu et al. (1993) reported that leaf water potential could have a role in the control of stomatal sensitivity to ABA.

A recent study of the effects of SIOS-induced water deficit stress on g_s and water relations in *Malus domestica* has shown that stomatal control may require modified water status in the leaf. The sensitivity of stomatal response to changes in leaf water status seemed to depend on the rate of water deficit induction (Guak, 1998; chapter 3). Moreover, the after-effect of SIOS-induced water deficit stress on stomatal opening following relief of stress (rewatering) persisted significantly longer than for water-stressed controls, although leaf turgor fully recovered to the prestress level. To better understand this effect on stomatal behavior, time-sequence measurements of leaf ABA

are presented here. The objectives of this study were to evaluate the effect of SIOS on the accumulation of ABA in the apple leaves, and to determine the relationship between stomatal behavior, bulk leaf ABA, and leaf water status.

4.3 Materials and Methods

4.3.1 Plant material and stress imposition

Bench-grafted 'Gala'/M26 apple plants were individually potted in 3.8-liter plastic pots with a 1:1:1:1 (by volume) mix of pumice/soil/coarse sand/peat, grown in a lathhouse at the Department of Horticulture, Oregon State University, Corvallis. Osmocote (Sierra Co.), a slow-release fertilizer (20-20-20 plus micronutrients), was incorporated into the medium at 5 grams per pot. Plants were trained to a single stem by removing axillary shoots.

On 19 August 1994, 21 days prior to the study, 160 uniform plants were transferred to a greenhouse under day/night temperatures of 28/18C, with natural light and photoperiod. Plants were arranged on a bench in a randomized complete-block design with 13 single-plant replicates per treatment (13 sampling dates), blocked according to light distribution. Forty plants in a block were spaced in four rows of ten plants with 30 cm between rows and 30 cm between trees. Extra five trees were used as guard plants for each end side. Leaves were limited to 20 per stem.

The potting media were brought to field capacity before treatment. They were treated with either water or sorbitol solution ($\approx$ -1.2 MPa osmotic potential) as a drench to the planting medium at rate of 400 ml per pot, and thereafter not watered for seven days,

serving as 'water-stressed control' and 'sorbitol-treated plant', respectively. One group was watered daily to field capacity throughout the experiment for 'well-watered control'. After the seven-day drying period, all trees were rewatered to field capacity for six consecutive days. The volumetric soil water content was determined with time domain reflectometry (TDR) (Fig. 4-1), as previously described (Guak, 1998; chapter 3).

4.3.2 Stomatal conductance and water relations

Stomatal conductance (g_s) was measured from fully expanded leaves (10-12 leaves from the top) with a Li-1600 steady-state porometer (LI-COR, Inc., Lincoln, NE). The leaves were then excised and brought to the laboratory to determine water relations and leaf ABA content. Leaf total water potential (Ψ_w), osmotic potential (Ψ_s), and turgor potential (Ψ_p), were determined as previously described (Guak, 1998; chapter 3). The diurnal time course was measured from 10 a.m. to 6 p.m. Throughout the experimental period, Ψ_w, Ψ_p, Ψ_s, and g_s were assessed between 11:00 a.m. and 12:00 p.m. at 1- to 3-day intervals with four replications per sampling date.

4.3.3 Extraction methods for radioimmunoassay analysis of bulk leaf ABA

Immediately after each Ψ_w measurement, a half-portion of the fully expanded leaf (midrib excluded) was wrapped in aluminum foil, frozen in liquid N_2, and stored at -80C. Leaves were freeze-dried, ground to a fine powder with a mortar and pestle, and put through a #40 mesh to eliminate leaf fibers. ABA was extracted by shaking 20-mg ground leaf samples in 1 ml of ddH_2O in eppendorf vials for 24 hr in a dark room at 4C. Fisher Vortex "Genie 2' holding 60 vials was used at shake level 3.

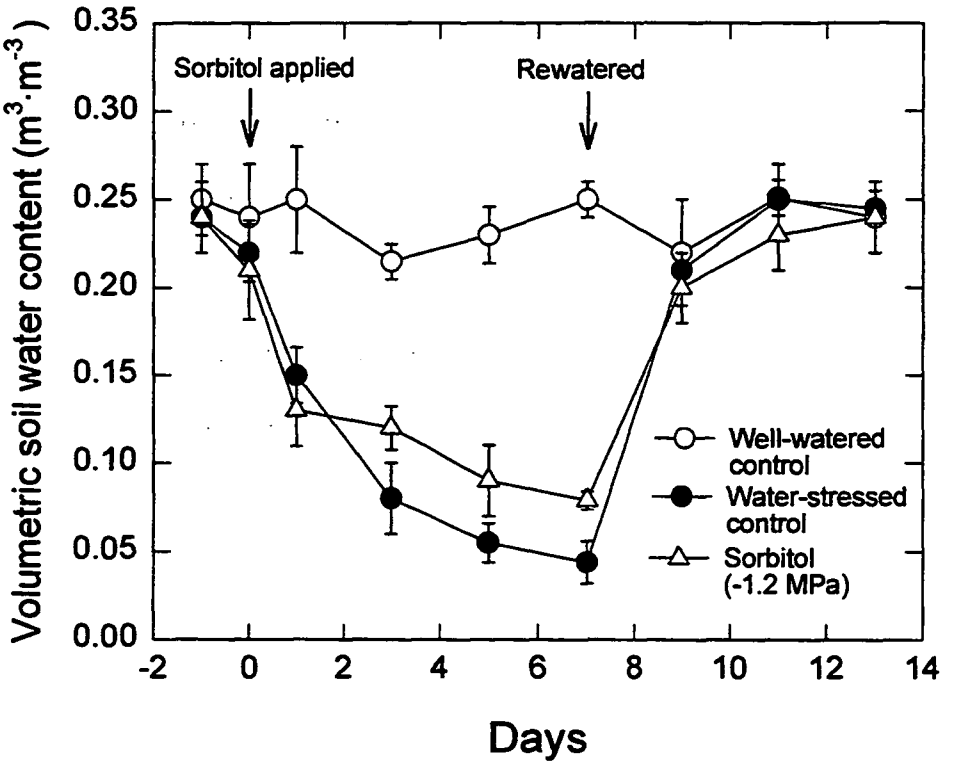

Figure 4-1. Volumetric soil water content measured with time domain reflectometry (TDR) during periods of drying and recovery from water stress. Values are mean ± SE (n=3).

4.3.4 RIA procedure

ABA concentration was measured according to Vernieri et al. (1989). A PBS buffer (50 mM sodium phosphate and 100 mM NaCl; pH 7.0) was used. Briefly, [G-^{3}H]-(RS)-Abscisic acid (approximately 2.5 Tbq•mmol^{-1}; Nycomed Amersham Place, Buckinghamshire, England, UK) in ethanol was diluted to 0.27 ml•ml^{-1} in PBS containing 5.0 mg•ml^{-1} bovine γ-globulin (Sigma Chemical CO., St. Louis, MO). This acted as a co-precipitant with the antibody. The antiserum (Sigma) developed in rabbit by using abscisic acid-HAS as the immunogen was reconstituted and diluted in PBS containing 5.0 mg•ml^{-1} bovine γ-globulin per supplier's directions. Samples and ABA standards were prepared in ddH_2O. Standard ABA solutions were made with (±) cis, trans-ABA (Sigma).

Solutions were added to 1.5 ml eppendorf assay vials in the following order: 50 µl of extracted sample, ABA standard or ddH_2O for B_{max} (100% binding of ABA tracer) determination, 100 µl [^{3}H-ABA] solution and 50 µl antibody solution. Non-specific binding (B_{min}) was determined by omitting antibody from the assay mixture. The contents were vortexed gently and incubated for 30 min at 4C. A saturated solution (200 µl) of ammonium sulfate (Grade 1, Sigma) was added to precipitate antibodies, and the mixture was incubated at room temperature in the dark for 30 min. The precipitated antibodies were then pelleted for 6 min at 13,000 g in an eppendorf microcentrifuge. The supernatant was discarded and the pellet washed by completely resuspending it in 400 µl of 50% saturated ammonium sulfate solution, then centrifuging for 6 min and again discarding the supernatant. After removing as much supernatant as possible, the pellets were completely dissolved in 100 µl ddH_2O; then a 1.2-ml cocktail solution (OPTI-

FLUOR, Packard Instrument Co., Meriden, CT) was added, and the samples were counted twice in a liquid-scintillation counter (Packard Tri-Carb 1900CA, Packard Instrument Co.) for 10 min each time.

ABA concentrations were calculated from the radioactivity present in the pellets. A series of six ABA standards (in two-fold dilutions from 13,000 to 130 pg per vial) was made for each batch of assays to construct a calibration curve (Fig. 4-2). This was linearized by subtracting B_{min} and plotting logit-transformation of the corrected data against the Ln of unlabelled ABA present per vial, where logit $(B/B_{max}) = Ln\ [(B/B_{max})/(1-B/B_{max})]$ and B was the corrected dpm bound in the presence of ABA standard. Sample ABA concentrations were interpolated from this line.

To test for possible non-specific interference from substances in the crude extract, we conducted ABA recovery controls. Such a control consisted of adding a range of authentic ABA standards to the plant sample dilutions and checking that the amounts of ABA measured by RIA increased in proportion to the ABA amounts added (Pengelly, 1985; Zhang and Davies, 1990). Any deviation from the slope of ABA standards alone caused by the addition of tissue samples would indicate interference (Fig. 4-3). The reported ABA data in this study were not corrected for contamination and were expressed on a dry-weight basis.

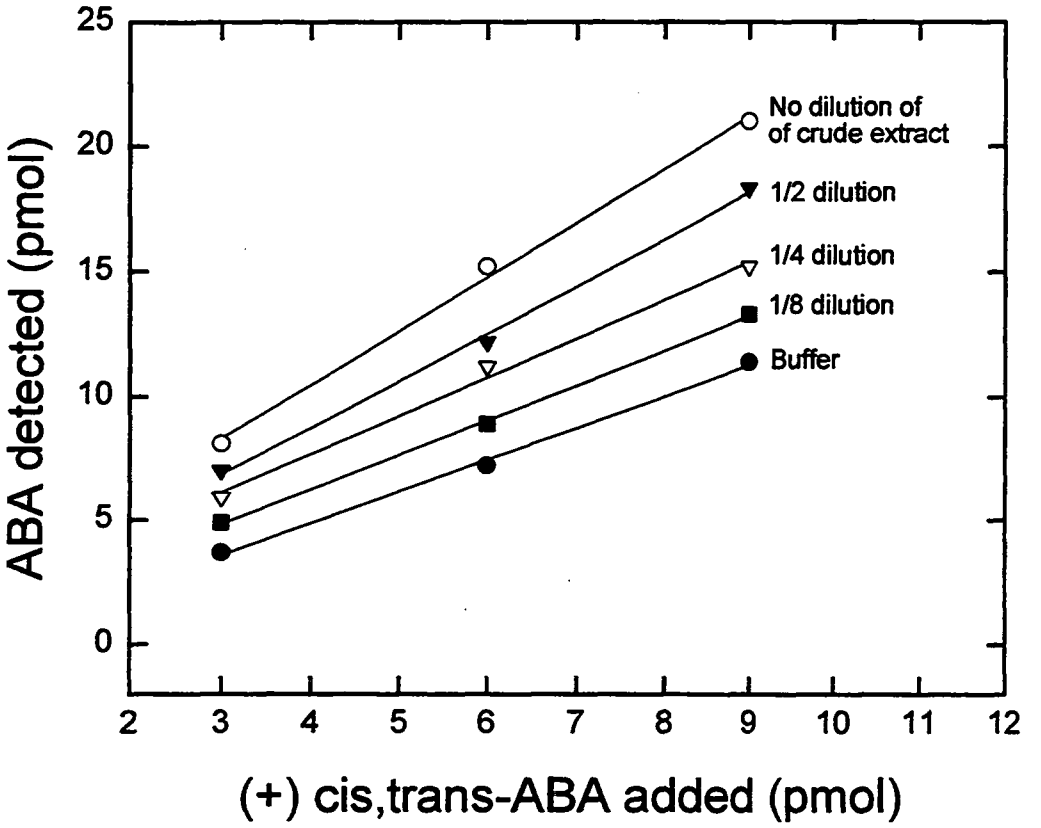

Figure 4-2. Standard curves for the RIA with (±) cis, trans-ABA and its linear transformation, showing two standard curve determinations. The inset shows logit of B/B_{max} = Ln $[(B/B_{max})/(1-B/B_{max})]$. B_{max} and B express 100% binding of ABA tracer and binding of ABA tracer in presence of sample, respectively. The mean R^2 for the linear regression was 0.99.

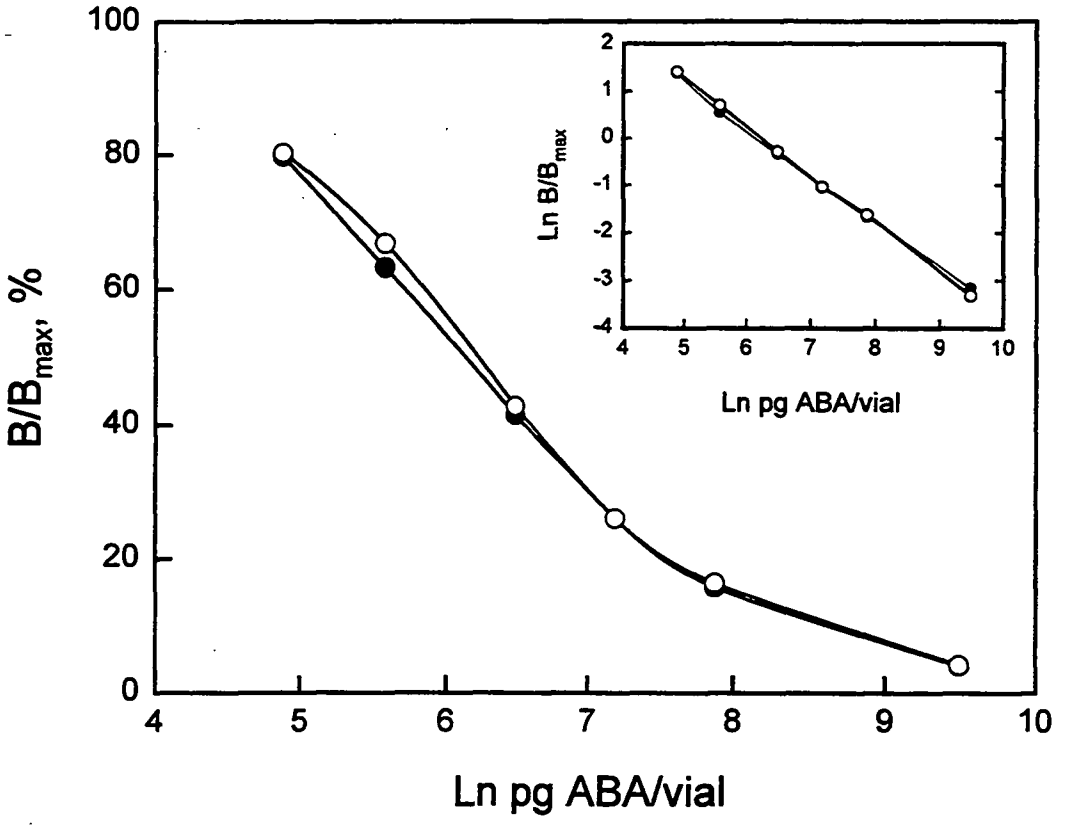

Figure 4-3. Internal standardization at various dilutions of (stressed) crude apple (*Malus domestica* Borkh. 'Gala'/M26) leaf extract.

4.4 Results

4.4.1 Changes in ABA and water relations in response to sorbitol-induced osmotic stress

The results on the time course of changes in Ψ_w, Ψ_p, g_s, and bulk leaf [ABA] in mature leaves of apple showed that sorbitol-induced osmotic stress (SIOS) treatment induced a rapid decrease in Ψ_w, Ψ_p, and g_s, and an increase in bulk leaf [ABA] was readily observed after two hr of treatment (Fig. 4-4a). SIOS treatment significantly increased leaf [ABA] to about 930 $ng.g^{-1}$ dry weight two hr after treatment. In well-watered control, there were no diurnal fluctuations of leaf [ABA] in well-watered plants; ABA content always ranged from 600 to 700 $ng.g^{-1}$ dry weight. The peak in leaf [ABA] of SIOS-treated plants gradually declined to the level of well-watered control 5 hr after treatment.

After a rapid increase on day 1 after treatment, the ABA level stabilized (Fig. 4-4b). On the first day, leaf [ABA] of well-watered controls were 600 to 700 $ng.g^{-1}$ dry weight, g_s was higher than 0.55 $cm.sec^{-1}$, and Ψ_w was -1.15 MPa (Fig. 4-4b). However, leaf [ABA] for the SIOS-treated plants peaked at about 900 $ng.g^{-1}$ dry weight on Day 1 after treatment whereas the water-stressed control leaf [ABA] peaked on Day 4 after treatment, when the soil began to dry out (Fig. 4-4b). These increases in leaf [ABA] were associated with the decline in g_s for the water-stressed control and the SIOS-treated plants. After leaf [ABA]reached a maximum on Days 1 and 5 for the SIOS-treated plants and the water-stressed control, respectively, g_s decreased to a minimum level.

The correlation between soil water content and bulk leaf [ABA] in water-stressed controls and SIOS-treated plants was negative (Fig. 4-5). The pattern of ABA increase

Figure 4-4a. Time course of changes in turgor potential Ψ_P, water potential Ψ_w, stomatal conductance g_s, and [ABA] in mature apple leaves of well-watered and sorbitol-treated plants during the first day of treatment. Values are means ± SE (n=3).

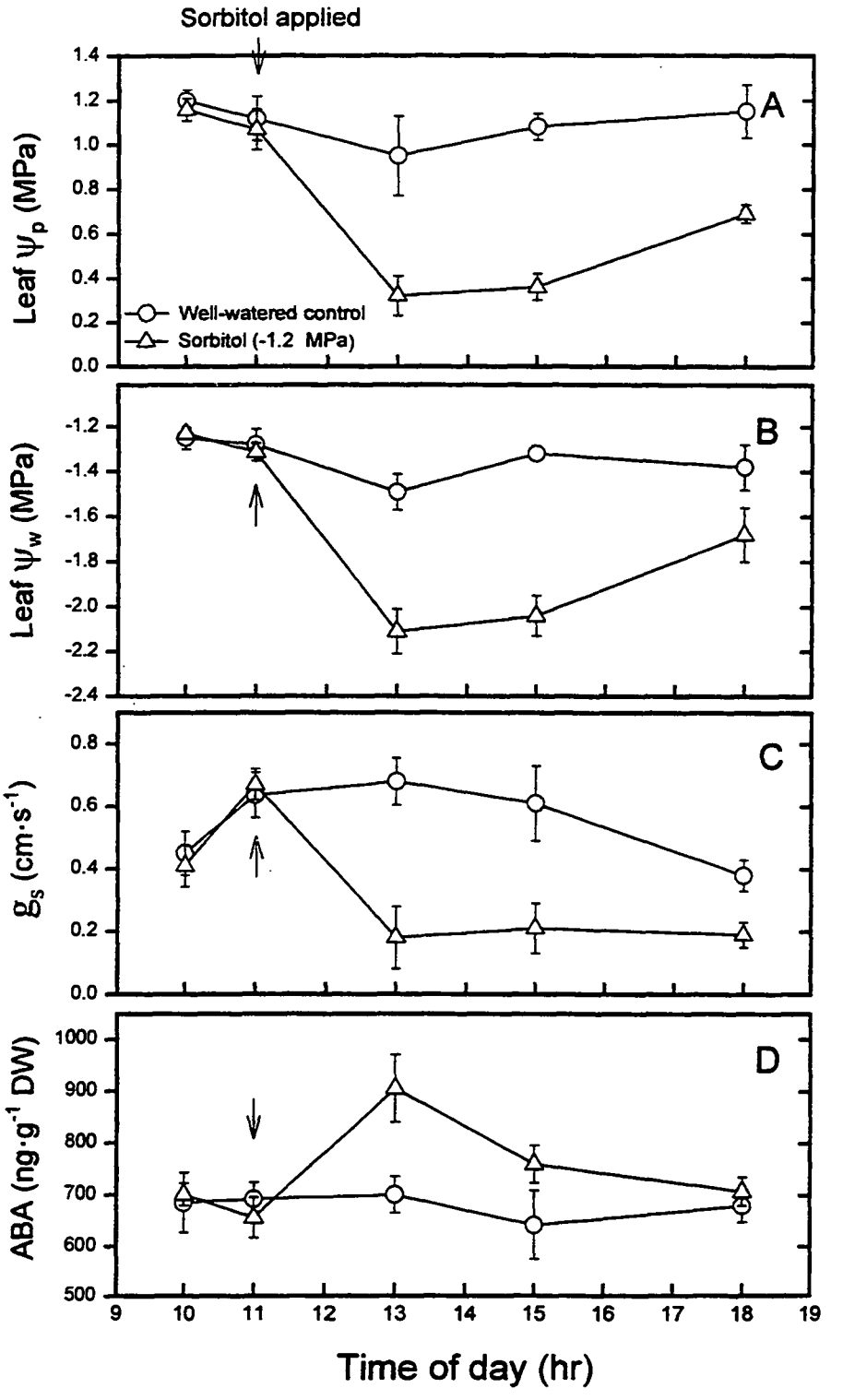
Sorbitol applied
A
B
C
D
Leaf ψ_p (MPa)
Leaf ψ_w (MPa)
g_s (cm·s^{-1})
ABA (ng·g^{-1} DW)
Well-watered control
Sorbitol (-1.2 MPa)
Time of day (hr)

Figure 4-4a

Figure 4-4b. Time course of changes in turgor potential Ψ_P, water potential Ψ_w, stomatal conductance g_s, and [ABA] in mature apple leaves of well-watered, water-stressed, and sorbitol-treated plants during periods of drying and recovery from water stress. Values are mean ± SE (n=3).

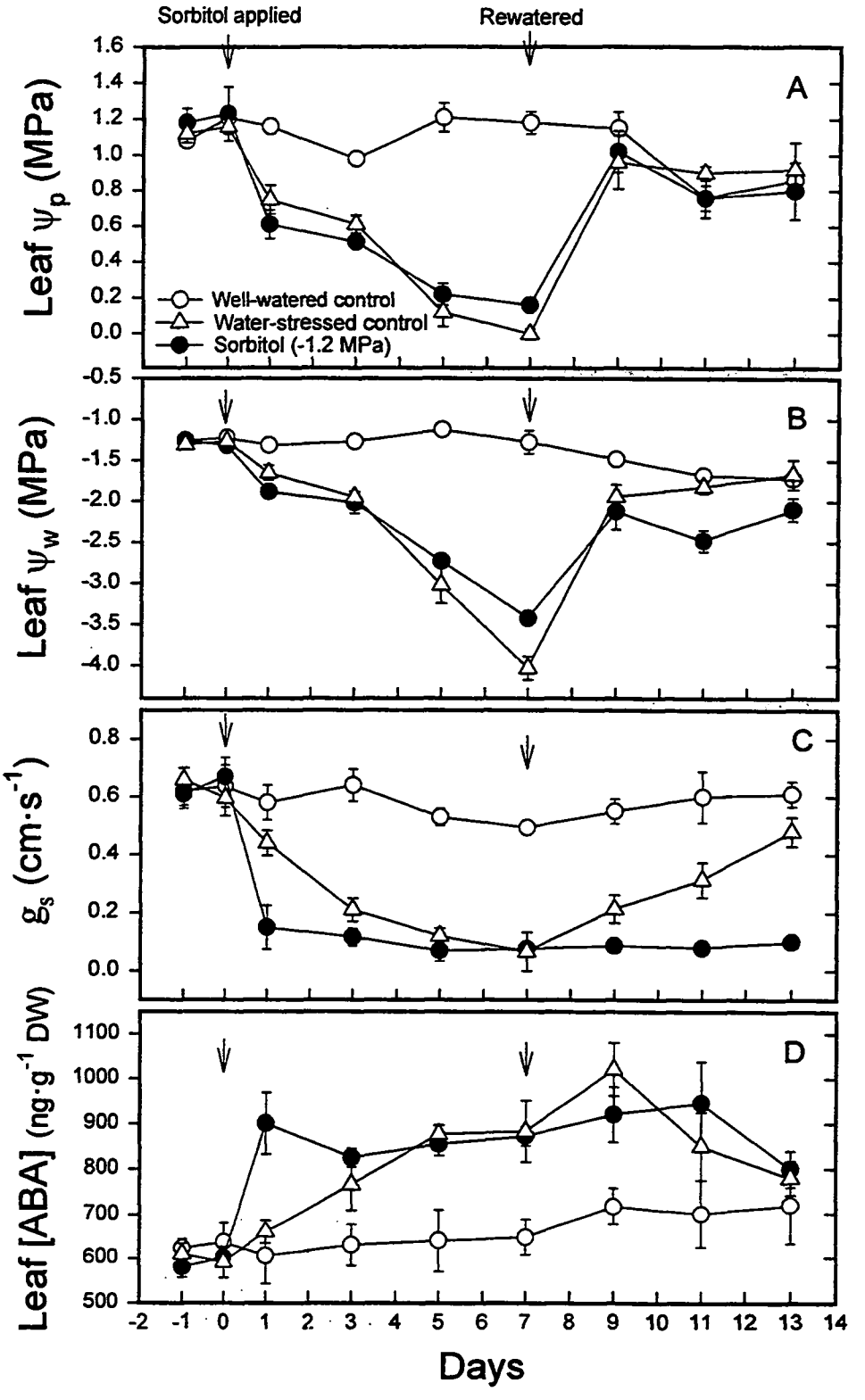
Sorbitol applied
Rewatered
A
B
C
D
Leaf ψ_p (MPa)
Leaf ψ_w (MPa)
g_s (cm·s^{-1})
Leaf [ABA] (ng·g^{-1} DW)
Well-watered control
Water-stressed control
Sorbitol (-1.2 MPa)
Days

Figure 4-4b

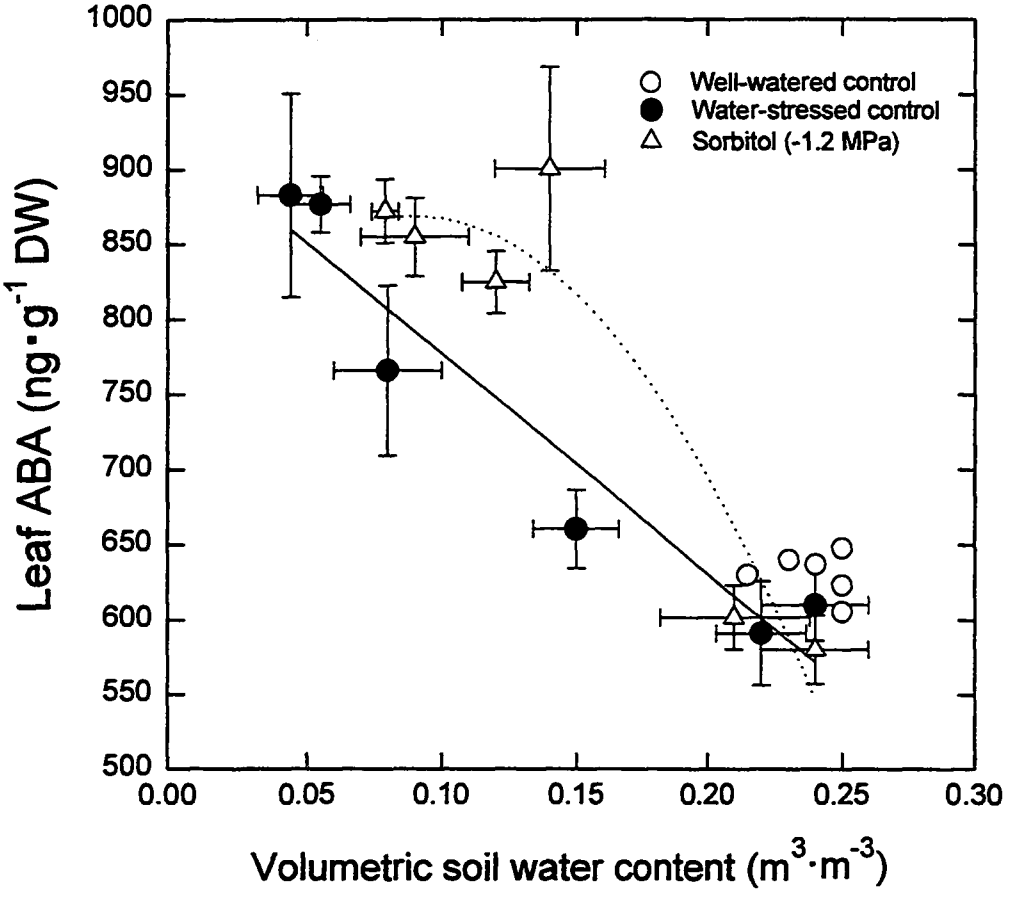

Figure 4-5. Relationship between leaf [ABA] and volumetric soil water content (θ) in apple treated with either water or sorbitol (-1.2 MPa) and exposed to a 7-day drying period. Regression equations: for water-stressed controls (solid line) leaf [ABA] = 924.25 - 1468.87θ (R^2 = 0.92***); for sorbitol-treated plants (dotted line) leaf [ABA] = 754.92 + 2539.75θ - 14186.54θ^2 (R^2 = 0.90***).

was specific for each treatment. For water-stressed controls, leaf [ABA] decreased linearly with decreasing soil water content and reached a maximum at 0.05 $m^3 \cdot m^{-3}$ of soil water content. In SIOS-treated plants the bulk leaf [ABA] reached a maximum much earlier, at 0.13 $m^3 \cdot m^{-3}$.

Ψ_w and Ψ_p were negatively correlated with bulk leaf [ABA] (Fig. 4-6). The accumulation of bulk leaf [ABA] as a function of Ψ_w and Ψ_P differed between water-stressed controls and SIOS-treated plants. For example, the maximum leaf [ABA] for the SIOS treatment was reached at the comparatively higher Ψ_p at 0.5 MPa while the water-stressed control reached a maximum when near zero. Likewise, SIOS-treated plants appeared to reach the maximum level of leaf [ABA] at the higher Ψ_w at -2.0 MPa than for the water-stressed controls at -3.0 MPa. Stomatal conductance showed a highly significant correlation with bulk leaf [ABA] or leaf Ψ_w (Fig. 4-7).

4.4.2 Changes in leaf water relations and levels of leaf ABA following recovery from water stress

Rehydration caused Ψ_p and Ψ_w of water-stressed control plants to return to pre-stress levels 2 days later (Fig. 4-4b). Turgor recovery for SIOS-treated plants was similar to that for water-stressed controls, but Ψ_w recovered slightly later.

After rewatering, leaf [ABA] slightly increased for 2-4 days, followed by a decrease to the level of well-watered controls. The decline in leaf [ABA] occurred 2 days earlier in water-stressed controls than in SIOS-treated plants; this was associated with a more rapid recovery of Ψ_w (Fig. 4-4b).

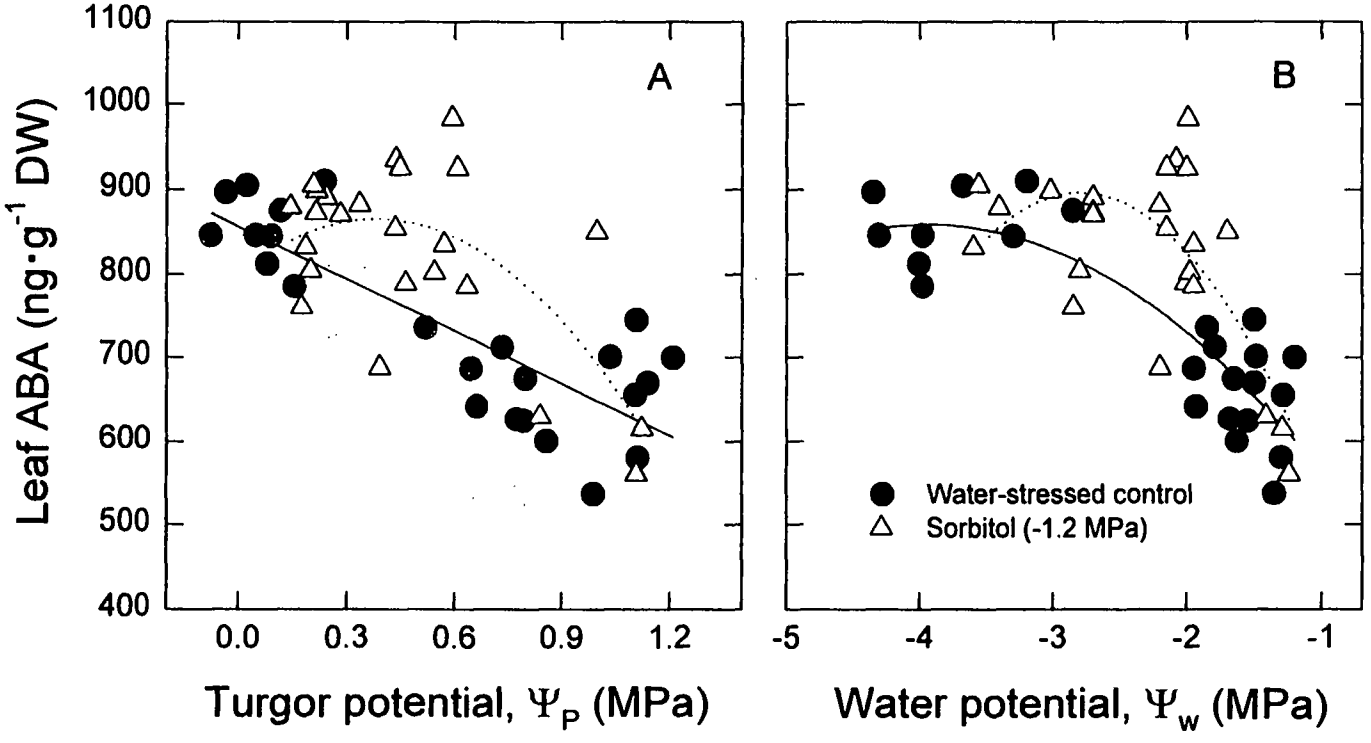

Figure 4-6. Relationship between leaf [ABA] and (A) turgor pressure, Ψ_P, and (B) water potential, Ψ_w, in apple treated with either water or sorbitol solution (-1.2 MPa) and exposed to a 7-day drying period. Regression equations: (A) for water-stressed controls (solid line), leaf [ABA] = 606.0 - 170.3Ψ_P (R^2 = 0.69**); for sorbitol-treated plants (dotted line), leaf [ABA] = 606.2 - 164.7Ψ_P (R^2 = 0.39*), and (B) for water-stressed controls (solid line), leaf [ABA] = 329.4 - 280.3Ψ_w - 37.0Ψ_w^2 (R^2 = 0.64**); for sorbitol-treated plants (dotted line), leaf [ABA] = 22.2 - 624.4Ψ_w - 111.5Ψ_w^2 (R^2 = 0.50**).

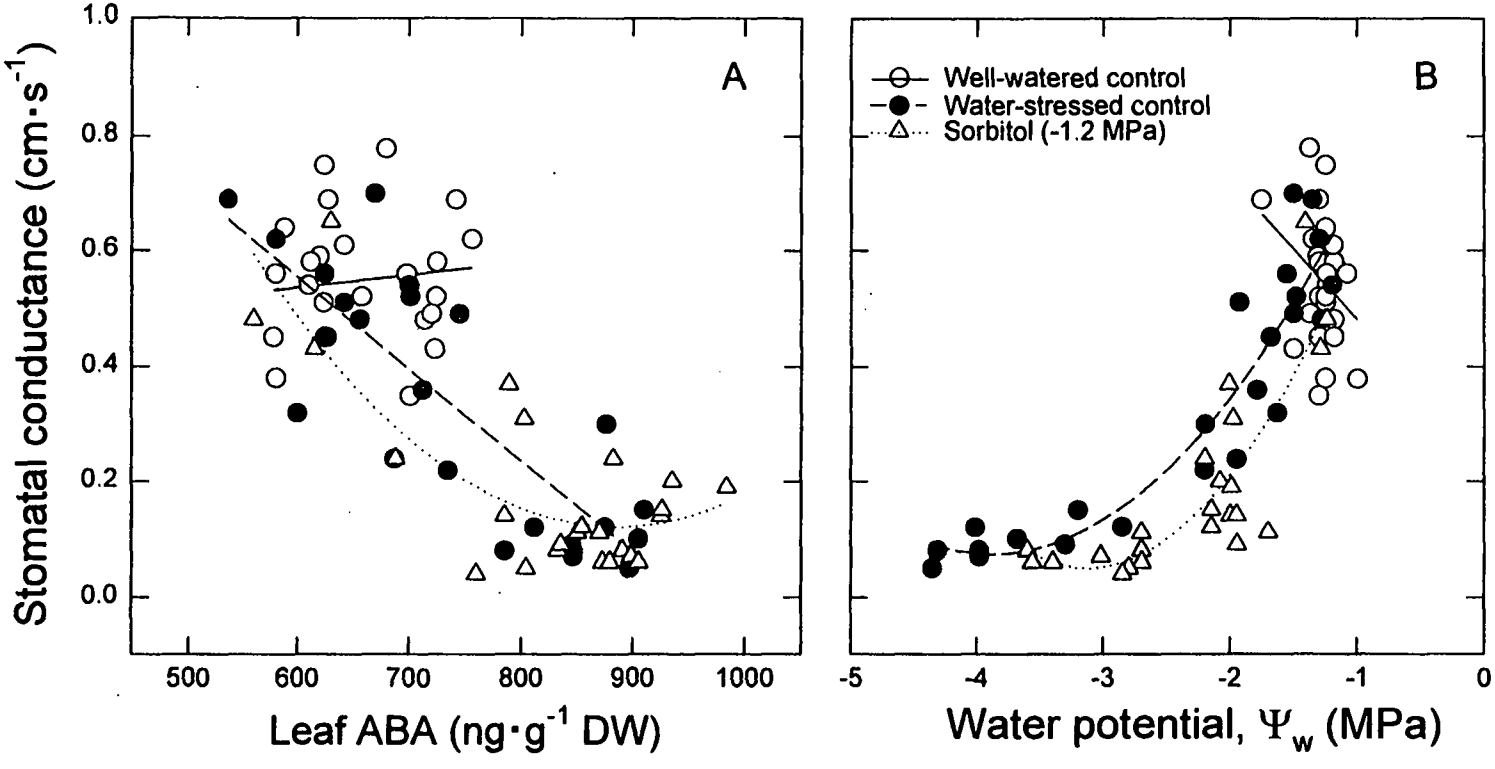

Figure 4-7. Relationship between stomatal conductance, g_s, and (A) leaf [ABA] and (B) leaf water potential, Ψ_w, in apple treated with either water or sorbitol solution (-1.2 MPa) and exposed to a 7-day drying period. Regression equations: (A) for well-watered controls (solid line), $g_s = 0.40 + 0.00022[ABA]$ ($R^2 = 0.013$); for water-stressed controls (short-dashed line), $g_s = 1.54 - 0.0016[ABA]$ ($R^2 = 0.71$**); and for sorbitol-treated plants (dotted line), $g_s = 3.63 - 0.0079[ABA] + 0.0000045[ABA]^2$ ($R^2 = 0.62$**); (B) for well-watered controls, $g_s = 0.234 - 2.452\Psi_w$ ($R^2 = 0.09$); for water-stressed controls, $g_s = 1.231 + 0.597\Psi_w + 0.077\Psi_w^2$ ($R^2 = 0.86$**); and for sorbitol-treated plants, $g_s = 1.296 + 0.799\Psi_w + 0.128\Psi_w^2$ ($R^2 = 0.69$**).

Stomatal conductance g_s in SIOS-treated plants did not recover during rewatering; g_s of water-stressed controls gradually recovered, though not to the pre-stress level. Stomata and ABA reacted more slowly than either Ψ_w and Ψ_p. Six days after rewatering g_s of water-stressed controls had recovered only to about 67% of the well-watered controls; in SIOS-treated plants, however, stomatal opening never occurred.

4.5 Discussion

4.5.1 Radioimmunoassay for ABA analysis

By using monoclonal antibodies ABA can often be measured directly in crude extracts (Quarrie et al., 1988; Walker-Simmons, 1987). It is often preferable to extract ABA from plant tissues with water so that organic solvents do not interfere with the immunoassay (Loveys and van Dijk, 1988; Quarrie et al., 1988). Apple leaf crude extracts in our study possibly contain some interfering substances, as seen by the unparalleled lines in Fig. 4-3. Quarrie et al. (1988) assayed cereal leaves and leaves of some dicotyledonous species such as lupins without purification. By adding compounds such as polyvinylpyrrolidone (Quarrie et al., 1988) problems arising from any phenols in the apple leaf crude extract can be alleviated (Rosher et al. 1985).

4.5.2 Changes in bulk leaf ABA in response to sorbitol-induced osmotic stress (SIOS)

Midday leaf [ABA] in apples were negatively correlated to midday Ψ_w, Ψ_p, and soil water content during the period of stress. In this scenario water stress first influences leaf Ψ_w and, simultaneously, its turgor Ψ_p. This in turn triggers the production of bulk

leaf ABA (Pierce and Raschke, 1980; Wright and Hiron, 1969). Compared to the water-stressed controls, SIOS treatment possibly induced a more rapid reduction in Ψ_p at a given Ψ_w because of the absence of osmotic adjustment (Fig. 4-6). This resulted in the higher rate of ABA production. Pierce and Raschke (1980), studying detached mature leaves of red kidney bean, showed that sensitivity in the production of ABA to changes in Ψ_w progressively increased as turgor approached zero. A similar observation was made in our study of mature apple leaves (Fig. 4-5): at a given Ψ_w (e.g., about -2.0 MPa) bulk leaf [ABA] was higher in SIOS-treated plants than in water-stressed controls. Such an increase in the sensitivity of ABA production to changes in Ψ_w following SIOS treatment may have been caused by the immediate decrease in Ψ_p, which would then trigger ABA synthesis in the leaves (Pierce and Raschke, 1980; Wright and Hiron, 1969).

Although SIOS treatment significantly increased leaf [ABA] even at relatively higher turgor pressure (i.e. at about 0.6 MPa) compared to the water stressed controls (Fig. 4-6), the apparent initial increase in ABA occurred when turgor dropped to about 0.3 MPa after two hr of treatment (Fig. 4-4a). That turgor recovered to about 0.6 MPa the next day with a slight further increase in ABA concentrations. Consequently, bulk leaf [ABA] of SIOS-treated plants exhibited a loose relationship with leaf turgor ($R^2 = 0.39$), compared to the relationship in water-stressed controls ($R^2 = 0.69$, $P<0.005$; Fig. 4-6).

4.5.3 Relationship between stomatal conductance and leaf water status

Our study confirmed the positive correlation between leaf water status (Ψ_w) and g_s in mature apple leaves as previously shown (Guak, 1998; chapter 3). This had also been reported in sunflower (Sadras et al., 1993a,b), sorghum (Sarig et al., 1988), and soybean

(Bennett et al., 1987). Schurr et al. (1992) and Zhang and Davies (1989) showed, however, that stomatal control in droughted sunflower plants was maintained without a change in Ψ_w or Ψ_p, and strongly suggested a role for xylem ABA. Tardieu et al. (1996) maintained that stomatal control depended on not only [ABA] in the xylem sap but also leaf water status that was controlled by g_s as a consequence of water flux through the plant.

Pooling the data in Fig. 4-7 for all treatments, the closure of stomata in mature apple leaves occurred over at least a 5- to 10-bar range of Ψ_w. This is broader than the 2- to 3-bar range in maize (Beadle et al., 1971; Fereres et al., 1978) and sorghum (Fereres et al., 1978). Woody tree species may be more capable of osmotic adjustment than are herbaceous species for maintaining turgor (Morgan, 1984). The sensitivity of stomatal closure to changes in Ψ_w seems to be greater in the SIOS-treated plants (from -1.3 to -2.0 MPa in Fig. 7) than for water-stressed controls (from -1.3 to -3.0 MPa). This difference may be attributed to different rates of osmotic adjustment between both treatments. The rapid imposition of sorbitol-induced water stress resulted in a rapid reduction in Ψ_p mainly through the reduction of Ψ_w ($\Psi_p = \Psi_w - \Psi_s$) while changes in Ψ_s were small.

4.5.4 Recovery from stress

The more rapid recovery of water-stressed control plants than SIOS-treated plants after rewatering found here confirms our previous results (Guak, 1998; chapter 3). Plants which had been stressed by SIOS treatment behaved differently than those with only water stress. When the water stress level was evaluated using only the leaf water status (e.g. Ψ_w), the condition of water-stressed control plants was more serious than for SIOS-

treated plants on Day 7 of treatment (Fig. 4b). Nevertheless, in the former the stomata gradually reopened after relief from stress (although the after-effect was manifested), whereas those in the latter remained closed even after Ψ_w and leaf [ABA] returned nearly to well-watered control levels. Because Ψ_w and Ψ_p had recovered rapidly in all leaves, the after-effect of water stress or SIOS-induced water stress cannot be linked to the reduced turgor. For the water-stressed controls, at least, the lag in recovery of g_s after rewatering might be due to the leaf [ABA] maintained at stress level for a few days after rewatering. This was not the case for SIOS-treated plants, because leaf [ABA] similarly changed in both treatments following rewatering. Mechanisms underlying the significant lag of g_s recovery from SIOS-treated plants may not be associated with ABA accumulation. Blake et al. (1991) and Levitt (1986) found that after-effect of water stress resulted from modification of leaf cell wall properties (e.g., elastic modulus).

After the relief of water stress, leaf [ABA] did not decline rapidly, but was maintained at the previous levels for 2-4 days, then declined slowly to the well-watered control level over 2-4 days. Such a decline of ABA levels in mature apple leaves following rehydration was not as rapid as in herbaceous species such as *Xanthium strumarium* (Zeevaart, 1980), possibly because, after stressed plants are rehydrated, xylem sap ABA trapped in root and xylem tissues during extended periods of water stress moves up into the leaves to increase or maintain leaf [ABA] by compensating for diluted (or metabolized) leaf [ABA]. It has also been suggested that the time of overall decline of leaf [ABA] depends on the rate of leaf ABA flux into the leaves (Gowing et al., 1993; Tardieu and Davies, 1993).

Inducing a longer after-effect on stomatal closure in plants through SIOS conditioning would be advantageous during future stress periods when excessive transpiration is prevented.

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CHAPTER 5

WATER RELATIONS, GAS EXCHANGE, AND ABA CONTENT OF THE LEAF AND XYLEM SAP IN APPLE (*Malus domestica* Borkh.) PLANTS IN RESPONSE TO SORBITOL-INDUCED OSMOTIC STRESS

5.1 Abstract

Bench-grafted 'Gala/M26' apple plants (*Malus domestica* Borkh.) were treated with either sorbitol (≈ -1.2 MPa osmotic potential) or water as a soil drench in outdoor condition of average 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ quantum flux, and thereafter water was withheld for eight days. Measurements of midday leaf water relations (water potential Ψ_w, osmotic potential Ψ_s, and turgor potential Ψ_p); gas exchange (stomatal conductance g_s, net photosynthesis *A*, and transpiration *E*); and [ABA] in the leaf and xylem sap were made to test the hypothesis that the rapid decrease in g_s following sorbitol-induced osmotic stress (SIOS) treatment is associated with a chemical signal (e.g., ABA) from the roots to the leaf (guard cells).

SIOS treatment resulted in rapid decreases (after two hr of treatment) in g_s, *A*, and *E* (55%, 33%, 36%, respectively) as well as decreased midday Ψ_w and Ψ_p (65% and 27%, respectively). Changes in [ABA] in the leaf and xylem sap, however, were not significant. Xylem sap [ABA] increased significantly from 27 to 38 $\mu mol \cdot m^{-3}$ after one day of treatment; leaf [ABA] increased significantly from 540 to 660 $ng \cdot g^{-1}$ DW after 3 days of treatment. These results suggest that a rapid stomatal response following SIOS treatment may result from rapid transmission of a hydraulic change from the root (rather than chemical signals like ABA). For water-stressed controls, significant decreases in Ψ_w, Ψ_s, and Ψ_p occurred in the first three days after water was withheld; however, g_s, *A*,

and *E* declined significantly three days later when the soil dried to about 30% of initial soil water content. This decrease in gas exchange corresponded to the significant increase in [ABA] in the leaf and xylem sap, suggesting the role of ABA in stomatal control in the water-stressed control.

5.2 Introduction

Plants regulate short-term water flow through the soil-plant-atmosphere continuum by controlling stomatal conductance. This adaptation is important in preventing critically low water potential and stress in the plant (Kramer and Boyer, 1995).

Conditioning plants with sorbitol can increase the desiccation resistance of plants by inducing stomatal closure (Guak, 1998; appendices B and C). Stomatal control is closely related to the leaf water status (water potential or relative water content), leaf turgor, and leaf [ABA] (Guak, 1998; chapter 4). These findings, however, were not sufficient to explain the cause of stomatal closure after sorbitol-induced osmotic stress (SIOS) treatment, such as chemical or hydraulic signals.

Studies, including split-root trials, have shown that as soil dries, stomatal conductance (g_s) decreases and leaf growth is reduced as a result of 'signals' produced by the roots and transported to the leaves (Bates and Hall, 1981; Davies and Sharp, 1981; Davies and Zhang, 1991; Gollan et al., 1985; Passioura, 1988). This has been documented in apple by Gowing et al. (1990). A close relationship has been shown between the concentration of ABA in the xylem sap and g_s both in the laboratory (Zhang and Davies, 1989a and 1990) and in the field (Wartinger et al., 1990; Tardieu et al.,

1992). However, there was a substantial difference in sensitivity of responses in the laboratory and in the field. Although many components of the xylem sap change in response to soil-drying (Gollan et al., 1992), ABA is generally considered to be the primary chemical involved in activating the 'signal' (Schurr et al., 1992; Zhang and Davies, 1989a). ABA fed to maize roots caused a substantial increase in xylem sap [ABA] and induced stomatal closure (Zhang and Davies, 1990), suggesting the role of root ABA in stomatal control. Hartung (1993) showed that ABA synthesized by the root tips could be transported to the apoplast next to the guard cells, the site of ABA action in stomata.

Most of the work examining drought-induced stomatal closure has been done with herbaceous species. In cowpea (Bates and Hall, 1981), in wheat (Gollan et al., 1986), in sunflowers (Zhang and Davies, 1989a,b, and 1990), and maize (Zhang and Davies, 1991), a close relationship between ABA in the xylem sap and g_s was observed. In one of the few studies involving woody plants, Khalil and Grace (1993) reported that g_s in sycamore maple seedlings (*Acer pseudoplatanus* L.) was related to [ABA] in the xylem sap, with no significant perturbation in shoot water status. The increase in root ABA was associated with the increase in xylem sap ABA. Henson and Turner (1991) showed that the level of ABA in the leaf was proportional to that produced by the roots of three lupin species. In contrast, Saliendra et al. (1995) suggested that in *Betula occidentalis* seedlings, it was hydraulic signals that probably prompted stomatal control against water stress, rather than chemical messengers (e.g., ABA) originating in the roots. Any treatment or event that changed soil water potential or hydraulic conductance resulted in hydraulic signals from the roots. They maintained that these signals were received much

earlier than chemical messengers carried in the transpiration stream. Stomatal conductance was also positively correlated with the apparent hydraulic conductance of the soil-root-shoot pathway in woody species (Kuppers, 1984) and in sugarcane (Meinzer and Grantz, 1990).

In this current study, apple xylem sap was expressed from the belowground shank (including the whole root system) to determine xylem sap [ABA] by radioimmunoassay (Vernieri et al., 1989). Xylem sap [ABA] and g_s data were used to test the hypothesis that the rapid decrease in g_s following SIOS treatment is associated with the concurrent increase in the xylem sap ABA generated from the root and transported to the shoot through the xylem.

5.3 Materials and Methods

5.3.1 Plant material and stress imposition

Bench-grafted 'Gala/M26' apple plants were potted in 3.8-liter plastic pots containing a 1:1:1:1 (by volume) mix of pumice, soil, coarse sand, and peat. They were grown in a lathhouse in Corvallis, Oregon. Plants were fertigated weekly with 200 ppm Peter's (20-20-20 with micronutrients) starting from May 10, 1995, and trained to single stems by removing axillary shoots. Trees (selected by size) were transferred to full sunlight on August 17, 1995, 21 days before beginning the experiment, and arranged in a randomized complete block design (3 blocks with 15 single-replicate trees each). Until the experiment was initiated, the plants were grown on saucers (#8) to collect irrigation water and prevent water stress.

One third of the trees were watered each morning and remained on saucers throughout the experiment (well-watered control); one third were watered on Day 0 (400 ml per pot), then left to dry for the next eight days (water-stressed control); and one third were soil-drenched with a sorbitol solution (≈ -1.2 MPa osmotic potential; 400 ml per pot) at 11:00 a.m. on Day 0 and then not watered for the next eight days [sorbitol-induced osmotic stress (SIOS) treatment].

5.3.2 Soil and leaf water status and leaf gas exchange measurements

Gravimetric soil water content was determined at 1- to 3-day intervals, from the midsection of the pot, when wet and dry (after 72 hr at 85°C) weights of soil samples were used. (Fig. 5-1).

Three plants per treatment, one per block, were measured on each sampling date. Leaf gas exchange and water potential were measured near midday (11:00-14:00 hr) on clear days at 1- to 3-day intervals, starting one day before the first treatment. On Day 0, measurements were taken two hr after treatment. Quantum flux densities during measurement averaged 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Gas exchange (stomatal conductance g_s, net photosynthesis A, and transpiration E) of the most recent, fully expanded leaves (upper 7 to 10) was measured with an open CIRAS-1 gas exchange system (PP Systems, Haverhill, MA).

After gas exchange measurements, the leaves were excised, placed in an insulated box with ice, and transported to the laboratory to determine leaf water relations, relative water content (RWC), and leaf [ABA]. The overall time from sample collection to water potential and RWC measurements was less than 10 min.

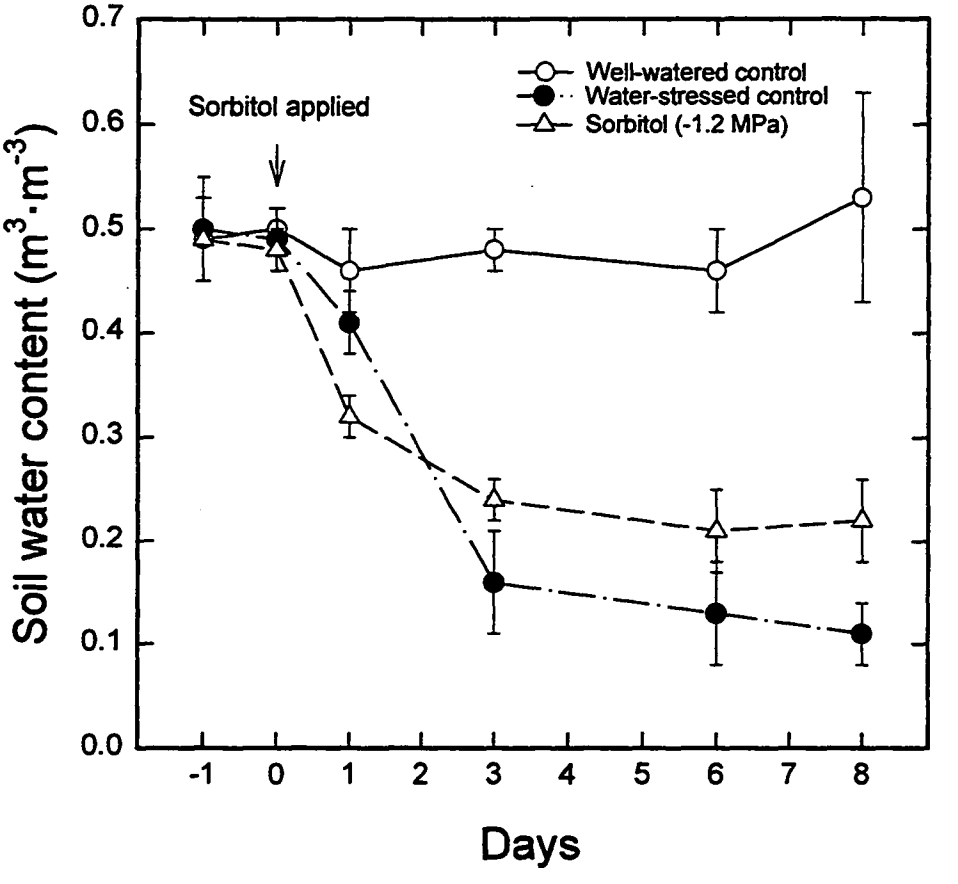

Figure 5-1. Time course of changes in gravimetric soil water content as drenched with water or sorbitol (-1.2 MPa). After drench, water was withheld throughout the experiment. Values are means ± SE (n=3).

Leaf water potential (Ψ_w) was measured with a pressure chamber (PMS Instruments, Corvallis, OR). Independent studies have shown the pressure chamber to be more accurate than a psychrometer (Jensen et al., 1989). Afterwards, each leaf was divided in two; one half was wrapped in aluminum foil to later determine leaf [ABA]. The other halves were put in 1.5-ml eppendorf tubes for determining osmotic potential (Ψ_s). All those samples were frozen in liquid nitrogen and stored at -80°C. For determining Ψ_s, these samples were smashed thoroughly for 1 min with a glass rod in the eppendorf tubes. A few drops of sap were decanted into clean tubes and centrifuged at 12,000 g for 5 min to obtain a clear sap. The 8-µl sap was micropipetted onto a filter paper disk in a vapor pressure depression osmometer (Wescor 5100C, Wescor Inc.) and osmolality was determined (Pomper and Breen, 1996). Osmolality was then converted to osmotic potential (Unit: MPa at 25°C) by using 2.48 MPa per Osmol•kg^{-1} (i.e., Ψ_s = -Conc•RT at 25°C = Osmol x 2.48, where R is the gas constant and T is the temperature in degree Kelvin).

5.3.3 Leaf extract and xylem sap sample preparations

The freeze-dried leaf samples for ABA analysis were finely ground with a mortar and pestle, extracted overnight in ddH_2O at 4°C, and microcentrifuged at 13,000 g for 6 min.

Xylem sap was collected from belowground shank tissues (including the whole root system) at 1- to 3-day intervals. Plants were bare-rooted without additional water; excess water was blotted from the roots (mostly for well-watered controls). The shank was cut about 5 cm above the most basal adventitious roots. Approximately 3 cm of bark

was removed from the top portion of the shank to prevent contamination of xylem and phloem sap. The root system was placed in the pressure chamber with the shank protruding. Pressures varied according to plant water status; 0.4 to 0.6 MPa and 1.0 to 2.5 MPa were applied to plants with little or no stress to mild or severe stress, respectively. Approximately 500 µl of sap was extracted from the former, whereas 200-300 µl of sap was collected from the latter. To prevent contamination from damaged surface cells the first few hundred µls were discarded (Anderson and Brodbeck, 1989; Wolf et al., 1990). The sap was collected in 1.5 ml eppendorf vials in shade, then frozen in liquid nitrogen, and stored at -80°C. Xylem sap was collected from three plants per treatment for each sampling date. Just before use, the sap was thawed and microcentrifuged at 12,000 g for 2 min to obtain clean sap.

5.3.4 Determination of ABA content with radioimmunoassay (RIA)

The ABA content of the leaf extract and xylem sap was determined by RIA (Vernieri et al., 1989) using a monoclonal antibody to ABA from Sigma (Sigma Chemical Co. St. Louis, MO). Briefly, a PBS buffer (50 mM sodium phosphate and 100 mM NaCl, pH 7.0) was used. Approximately 2.5 $TBq.mmol^{-1}$ [G-^{3}H]-(RS)-Abscisic acid (Nycomed Amersham Place, Buckinghamshire, England, UK) in ethanol was diluted to 0.27 $ml.ml^{-1}$ in PBS containing 5.0 $mg.ml^{-1}$ bovine γ-globulin (Sigma), a co-precipitant with the antibody. The antiserum (Sigma), developed in rabbit with abscisic acid-HAS as the immunogen, was reconstituted and diluted in PBS containing the co-precipitant, per supplier's directions. Standard ABA solutions were prepared with (±) cis, trans-ABA (Sigma).

Solutions were added to 1.5 ml eppendorf assay vials in the following order: 50 µl of leaf extract or xylem sap, ABA standard or ddH_2O for B_{max} (100% binding of ABA tracer) determination, 100 µl [^{3}H-ABA] solution, and 50 µl antibody solution. Non-specific binding (B_{min}) was determined by omitting antibody from the assay mixture. The contents were vortexed gently and incubated for 30 min at 4°C. A saturated solution (200 µl) of ammonium sulfate (Grade 1, Sigma) was added to precipitate antibodies, and the mixture was then incubated at room temperature in the dark for 30 min. The precipitated antibodies were pelleted for 6 min at 13,000 g in an eppendorf microcentrifuge. The supernatant was discarded and the pellet was washed by resuspending it completely in 400 µl of 50% saturated ammonium sulfate solution, centrifuging for 6 min and again discarding the supernatant. After removing as much supernatant as possible, the pellets were completely dissolved in 100 µl ddH_2O. A 1.2-ml cocktail solution (OPTI-FLUOR, Packard Instrument Co., Meriden, CT) was added, and the samples were counted twice in a liquid-scintillation counter (Packard Tri-Carb 1900CA, Packard Instrument Co.) for 10 min each time.

Generation of standard curves and the calculation of the ABA concentrations were described in detail in chapter 4 (Guak, 1998). Concentrations of ABA were calculated from the radioactivity present in the pellets. A series of six ABA standards (in twofold dilutions from 13000 pg to 130 pg per vial) was run in each batch of 28 samples for constructing a calibration curve. This was linearized by subtracting B_{min} and plotting logit-transformation of the corrected data against the Ln of unlabelled ABA present per vial, where Logit $(B/B_{max}) = Ln\ [(B/B_{max})/(1-B/B_{max})]$ and B is the corrected dpm bound in

the presence of ABA standard. Sample ABA concentrations were interpolated from this line. The measured ABA never fell outside the range of the standard curve. The average correlation coefficient (R^2) of the standard curve was 0.98 (n = 8). The recovery of ABA for the sample was not determined. The validation of RIA, for use with crude apple xylem sap, was confirmed by a dilution/spike recovery test for non-specific interference, as described by Jones (1987). Validation of RIA for use with crude extract from the leaves of apple plants was reported in chapter 4 (Guak, 1998). A pooled sample of crude xylem sap, undiluted or diluted to 50% and 25% with ddH_2O, was assayed in the presence of increasing amounts of synthetic (±) cis, trans-ABA standard (0.5, 2, and 4 pmol).

5.4 Results

5.4.1 Soil and plant water status

SIOS treatment caused a rapid decrease in soil water content, from 0.50 to 0.32 $m^3 \cdot m^{-3}$ one day after treatment (Fig. 5-1). Soil water content in the SIOS treatment remained constant at 0.25 H_2O $m^3 \cdot m^{-3}$ from Day 3 through the next five days, indicating little water uptake by plants during this period. In the water-stressed controls, however, most of the soil water was lost to 0.15 $m^3 \cdot m^{-3}$ by the third day of drying.

Ψ_w decreased rapidly from -0.80 to -1.32 MPa after sorbitol (-1.2 MPa) was applied, as measured two hr after treatment (Table 5-1). By Day 3, Ψ_w had further decreased to -2.0 MPa and thereafter remained relatively constant throughout the experiment (Fig. 5-2). In the water-stressed control, however, a significant decrease in Ψ_w (from -1.0 to -2.0 MPa) occurred between Day 1 and 3 as the soil dried, and by Day 8

Table 5-1. Effects on water relations (water potential, osmotic potential, and turgor potential), gas exchange (stomatal conductance and photosynthesis), and ABA content of the xylem sap and mature leaf of apple (*Malus domestica* Borkh. 'Gala'/M26), measured two hr after sorbitol (-1.2 MPa) treatment. Values are means ± (SE) (n=3).

Treatments	Potentials (MPa)			Stomatal conductance ($mmol \cdot m^{-2} \cdot s^{-1}$)	Photosynthesis ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Transpiration, ($mmol \cdot m^{-2} \cdot s^{-1}$)	Leaf [ABA] ($ng \cdot g^{-1}$ DW)	Xylem sap [ABA] ($\mu mol \cdot m^{-3}$)
	Water, Ψ_w	Osmotic, Ψ_s	Turgor, Ψ_p					
Well-watered control	-0.80 a[z]	-2.95 a	2.15 a	226.3 a	9.2 a	23.4 a	532.5 a	24.2 a
Sorbitol (-1.2 MPa)	-1.32 b	-2.90 a	1.58 b	101.6 b	6.2 b	14.9 b	551.7 a	27.6 a

[z]Different letters within column indicate significant level at $P = 0.05$, means separated by t-test.

decreased to -3.5 MPa. The Ψ'_s, measured two hr after treatment, was not affected by the SIOS treatment (Table 5-1). Ψ_s in the SIOS-treated plants decreased to about -3.3 MPa by Day 3 and remained constant for the next five days, whereas in the water-stressed plants it continued to decrease to -3.6 MPa after eight days. Ψ_s of well-watered plants remained between -2.9 and -3.2 MPa throughout the period.

A rapid decrease in Ψ_p with SIOS treatment was related with a rapid decrease in Ψ_w (Table 5-1). In the water-stressed controls, Ψ_p decreased significantly from Day 3, nearly reaching zero on Day 6 as the soil water depleted (Fig. 5-2). In contrast, the Ψ_p of the SIOS treatment remained above 1.0 MPa throughout the test period.

5.4.2 Stomatal conductance, photosynthesis, and transpiration

The g_s, *A*, and *E*, as measured two hr after SIOS treatment, decreased significantly (Table 5-1). On Day 1, g_s, *A*, and *E* in SIOS-treated plants decreased to 45%, 50%, and 48% of that for the well-watered controls, respectively (Fig. 5-2 and 5-3). Thereafter, these parameters in SIOS-treated plants dropped to their minimums on Day 6. In water-stressed controls, g_s, *A*, and *E* did not change significantly through Day 3. For the next three days, however, g_s, *A*, and *E* in the water-stressed controls decreased rapidly to minimum values. The pattern of change in g_s paralleled that in *A* or *E*, suggesting that *A* and *E* were largely dependent on g_s.

Stomatal conductance was highly correlated with Ψ_w, Ψ_p, and RWC in both water-stressed controls and SIOS-treated plants (Fig. 5-4). The pattern of correlation varied between the treatments. Complete closure of stomata occurred at much higher values of Ψ_w, Ψ_p, and RWC in SIOS-treated plants than in the water-stressed controls:

Figure 5-2. Time course of changes in stomatal conductance g_s, turgor potential Ψ_P, osmotic potential Ψ_s, and water potential Ψ_w in mature leaves of apple (*Malus domestica* Borkh. 'Gala'/M26) treated with either water or sorbitol (-1.2 MPa osmotic potential) as a soil drench. After treatment, water was withheld throughout the experiment. Values are means ± SE (n=3).

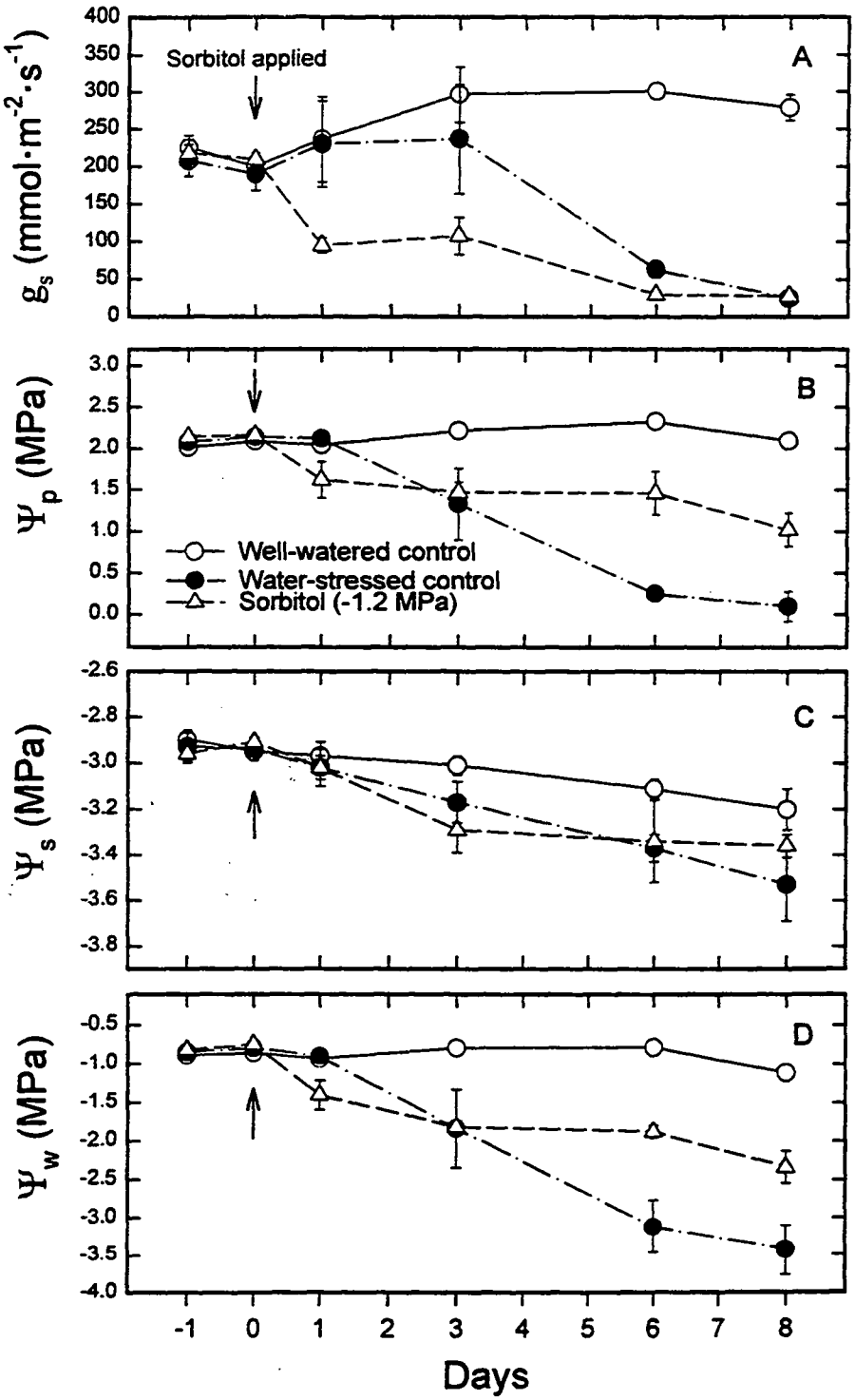
Sorbitol applied
A
B
C
D
g_s (mmol·m^{-2}·s^{-1})
Ψ_p (MPa)
Ψ_s (MPa)
Ψ_w (MPa)
Well-watered control
Water-stressed control
Sorbitol (-1.2 MPa)
Days

Figure 5-2

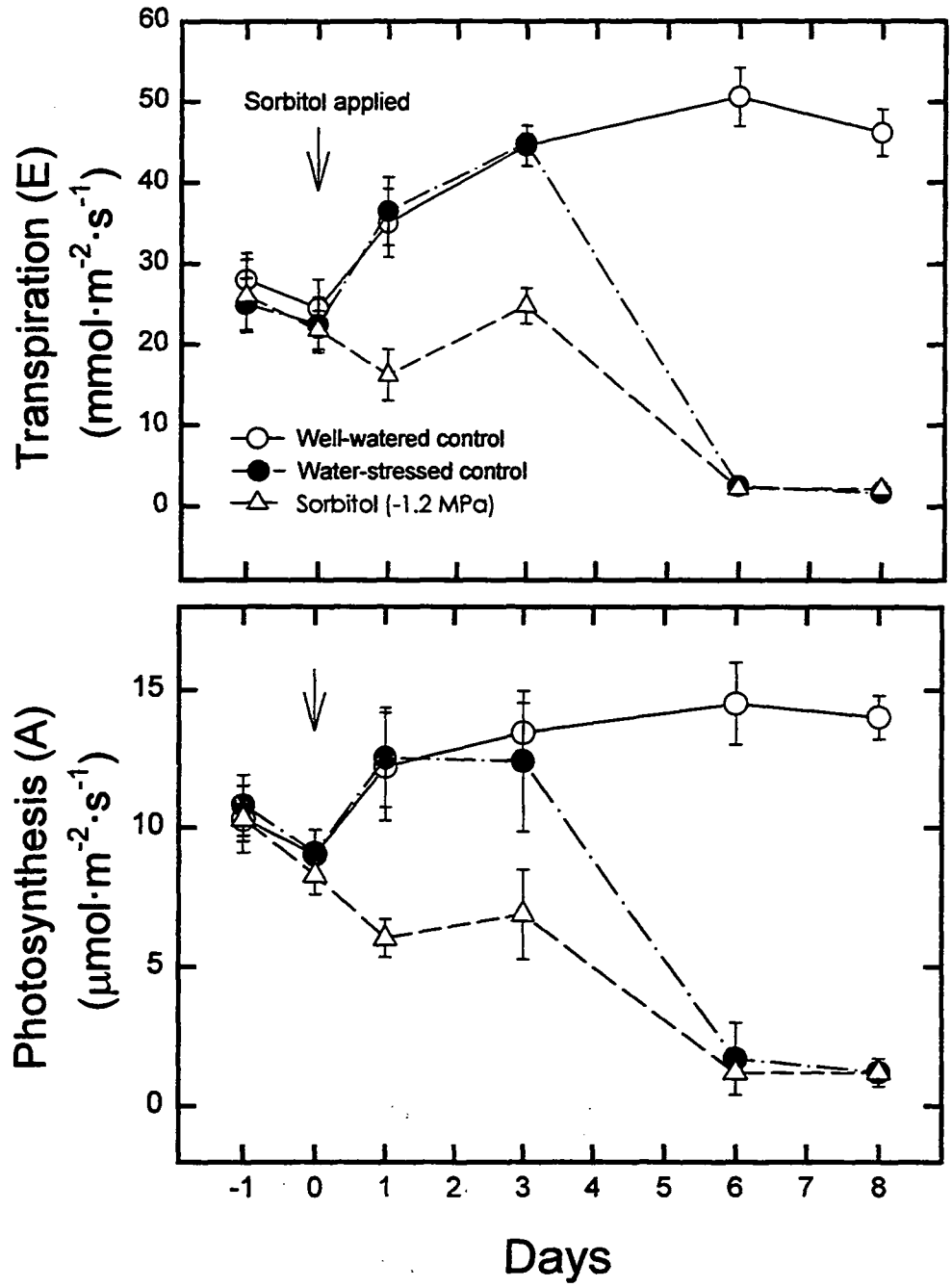

Figure 5-3. Time course of changes in the rates of transpiration and photosynthesis in mature leaves of apple treated with water or sorbitol (-1.2 MPa) as a soil drench. After treatment, water was withheld throughout the experiment. Values are means ± SE (n=3).

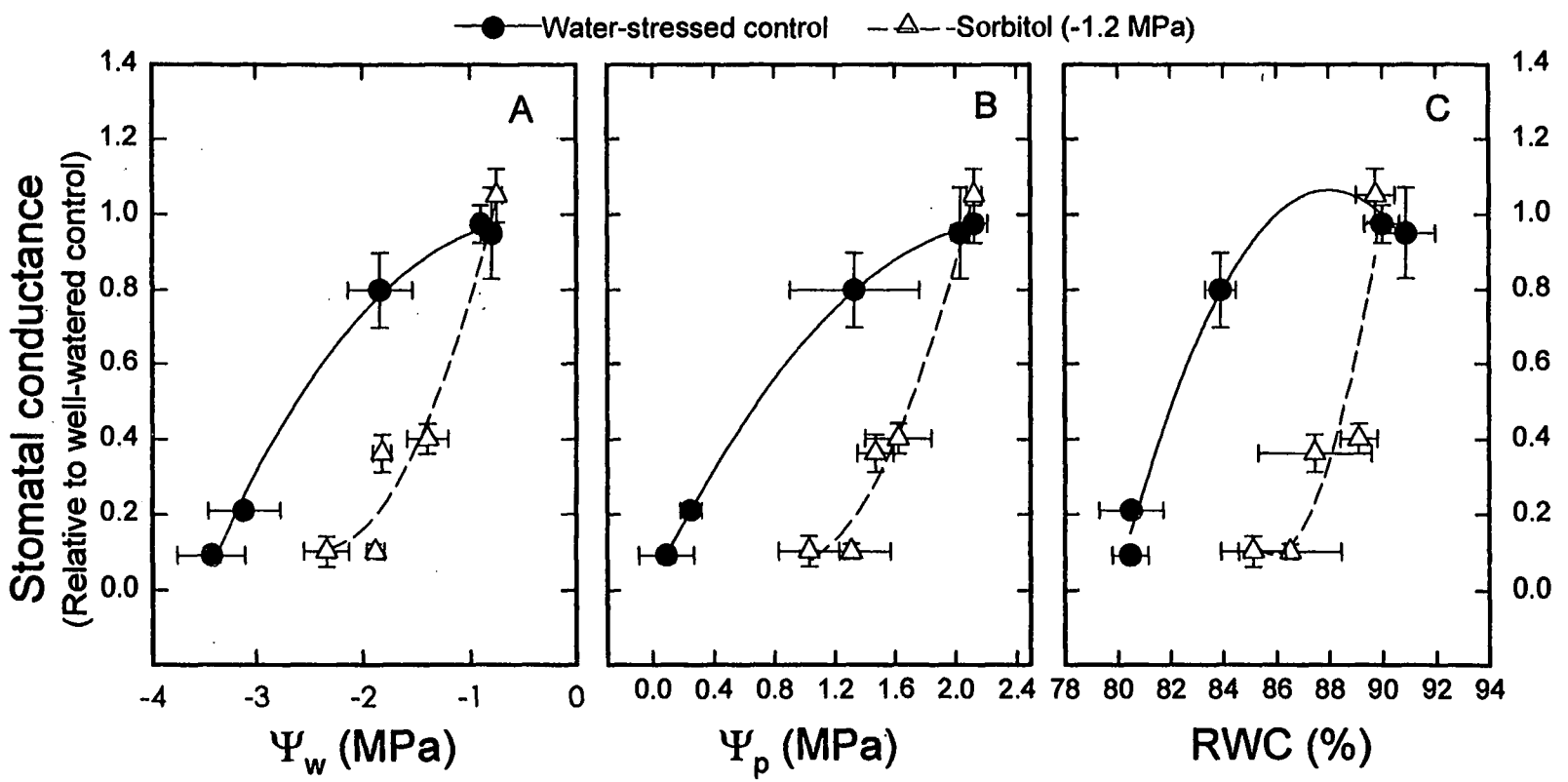

Figure 5-4. Relationship between stomatal conductance g_s, (A) water potential Ψ_w, (B) turgor potential Ψ_P, and (C) relative water content RWC for water-stressed controls and sorbitol-treated apple plants relative to well-watered control. Values are means ± SE (n=3). Regression equations: (A) water-stressed controls, $g_s = 0.95 - 0.100\Psi_w - 0.104\Psi_w^2$ ($R^2 = 0.99^{**}$); sorbitol-treated plants, $g_s = 2.09 + 1.66\Psi_w + 0.35\Psi_w^2$ ($R^2 = 0.95^{**}$); (B) water-stressed controls, $g_s = 0.017 + 0.82\Psi_P - 0.18\Psi_P^2$ ($R^2 = 0.99^{**}$); sorbitol-treated plants, $g_s = 0.60 - 1.179\Psi_P + 0.66\Psi_P^2$ ($R^2 = 0.98^{**}$); (C) water-stressed controls, $g_s = -124.04 + 2.84RWC - 0.016RWC^2$ ($R^2 = 0.99^{**}$); sorbitol-treated plants, $g_s = 389.77 - 9.079RWC + 0.053RWC^2$ ($R^2 = 0.82^{*}$). The significance of correlation coefficient was determined at df = 4.

stomata closed completely at an average of -3.5 and -2.0 MPa Ψ_w; at 0.1 and 1.2 MPa Ψ_p; and at 80 and 86% RWC in water-stressed controls and SIOS-treated plants, respectively.

5.4.3 Abscisic acid in leaf extracts and xylem sap

Figure 5-5 shows the relationship between ABA added and ABA detected for different dilutions. When the amount of ABA recovered was plotted against ABA added, the lines were fairly parallel between the ABA standard (without xylem sap) and the crude xylem sap. Therefore, the presence of non-specific interference was not significant in apple xylem sap.

No significant changes in [ABA] in the leaf and xylem sap of well-watered controls were found throughout the experimental period (Fig. 5-6). Leaf [ABA], measured two hr after SIOS treatment, was not affected (Table 5-1), but a significant increase in leaf [ABA] of SIOS-treated plants occurred by Day 3. Leaf [ABA] of water-stressed controls increased on Day 6 (approximately 700 $ng \cdot g^{-1}$ DW). These raised levels were maintained until the end of the drying period for both treatments (Fig. 5-6).

In the water-stressed controls, the significant decrease in g_s by Day 6 seemed to be related to the substantial increase in leaf [ABA]. In the SIOS-treated plants, however, the significant decrease in g_s after two hr of treatment (Table 5-1) or on Day 1 (Fig. 5-2) was not associated with changes in leaf [ABA]. Fig. 5-7 demonstrates that leaf [ABA] was negatively correlated with g_s changes in both treatments, even though the initial decline in g_s in response to SIOS was not related to leaf [ABA]. Stomatal conductance reached zero at 650 to 700 $ng \cdot g^{-1}$ DW, regardless of treatment. Stomatal sensitivity to leaf [ABA] was significantly higher in SIOS-treated plants than in water-stressed controls. A covariance

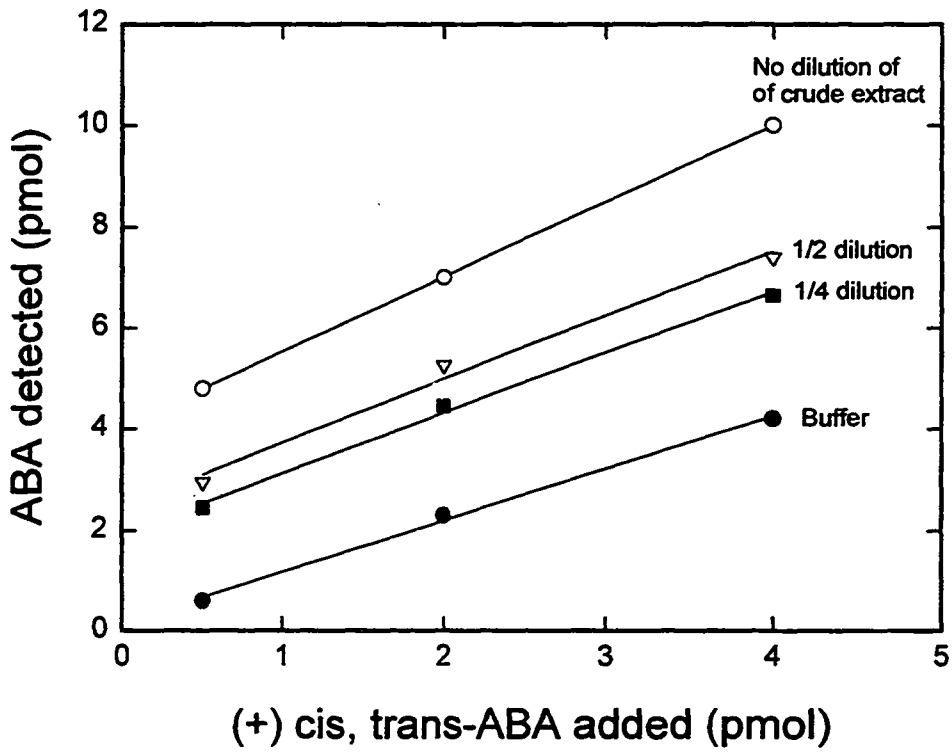

Figure 5-5. Internal standardization at various dilutions of crude xylem sap from stressed apple plants. A range of authentic ABA standards was added to sample dilutions.

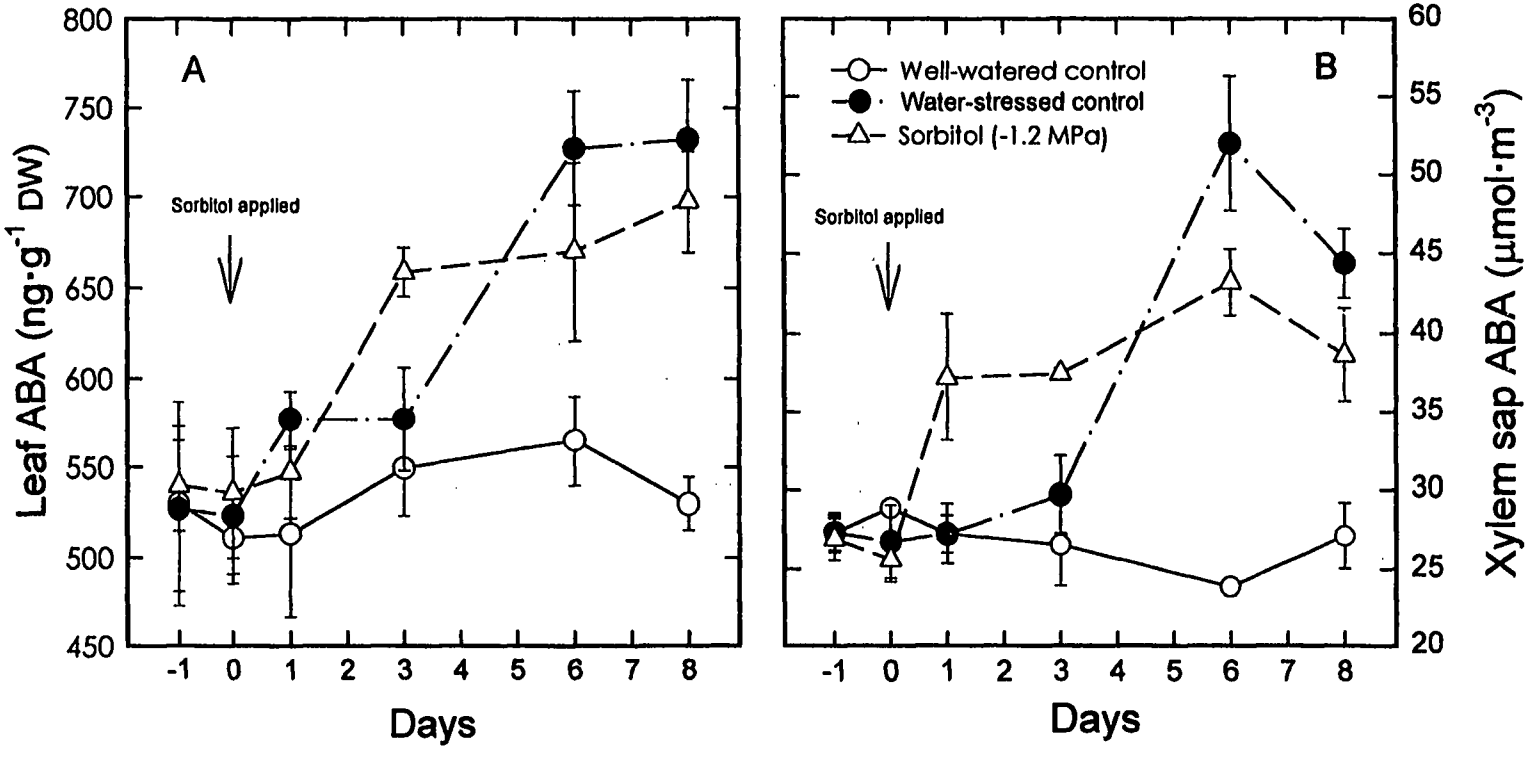

Figure 5-6. Time course of changes in leaf (mature) and xylem sap [ABA] in apple treated with either water or sorbitol solution (-1.2 MPa) as a soil drench. After treatment, water was withheld throughout the experiment. Values are means ± SE (n=3).

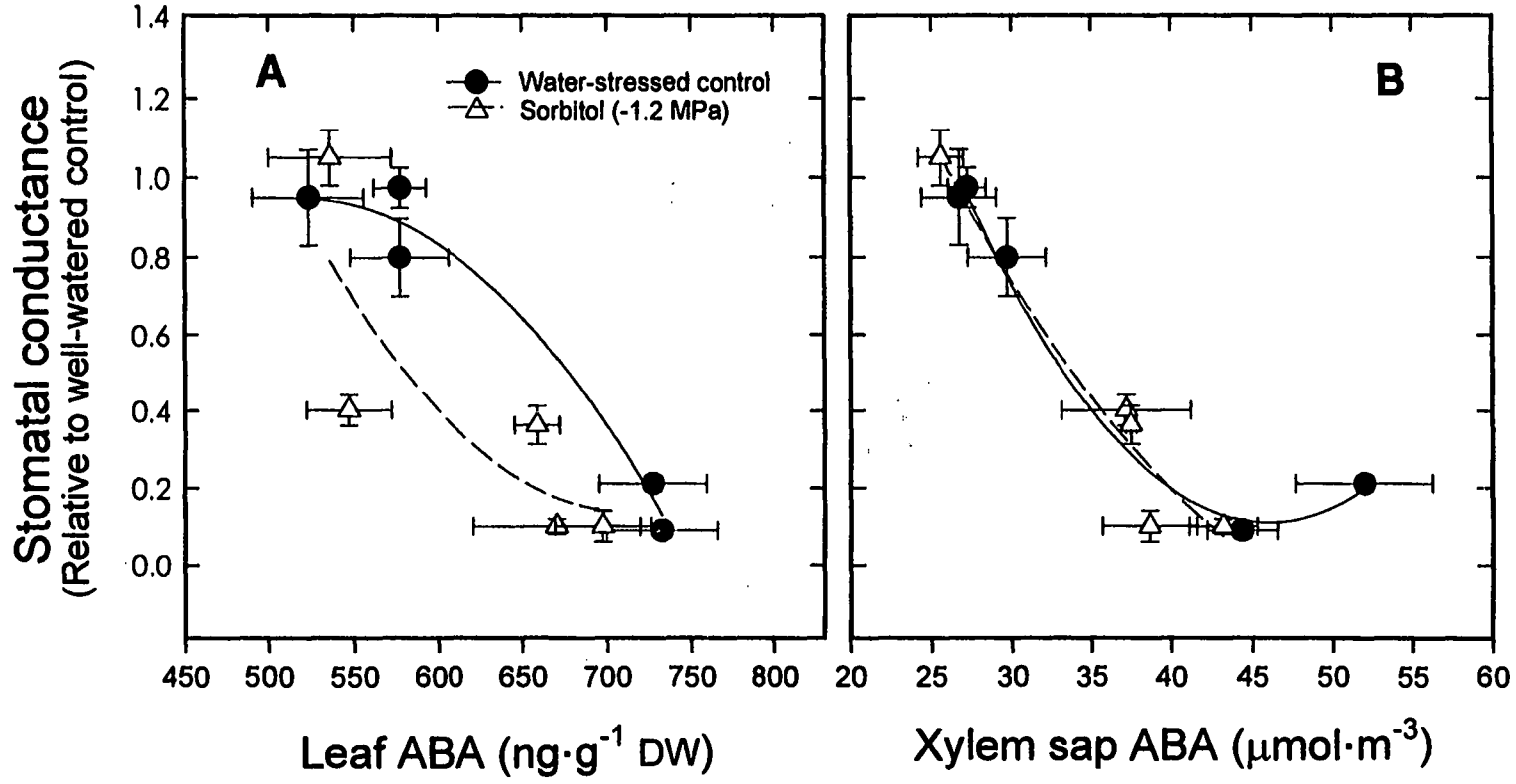

Figure 5-7. Relationship between stomatal conductance g_s, leaf [ABA] (A) and xylem sap [ABA] (B) in apple for water-stressed controls and sorbitol-treated plants relative to well-watered control. Values are means ± SE (n=3). Regression equations: (A) water-stressed controls, $g_s = -4.02 - 0.019[ABA] - 0.0000183[ABA]^2$ ($R^2 = 0.97^{**}$); sorbitol-treated plants, $g_s = 10.96 - 0.03[ABA] + 0.0000215[ABA]^2$ ($R^2 = 0.67^{*}$); (B) water-stressed controls, $g_s = 5.20 - 0.22[ABA] + 0.00241[ABA]^2$ ($R^2 = 0.99^{**}$); sorbitol-treated plants, $g_s = 3.84 - 0.14[ABA] + 0.00124[ABA]^2$ ($R^2 = 0.94^{**}$). The significance of correlation coefficient was determined at df = 4.

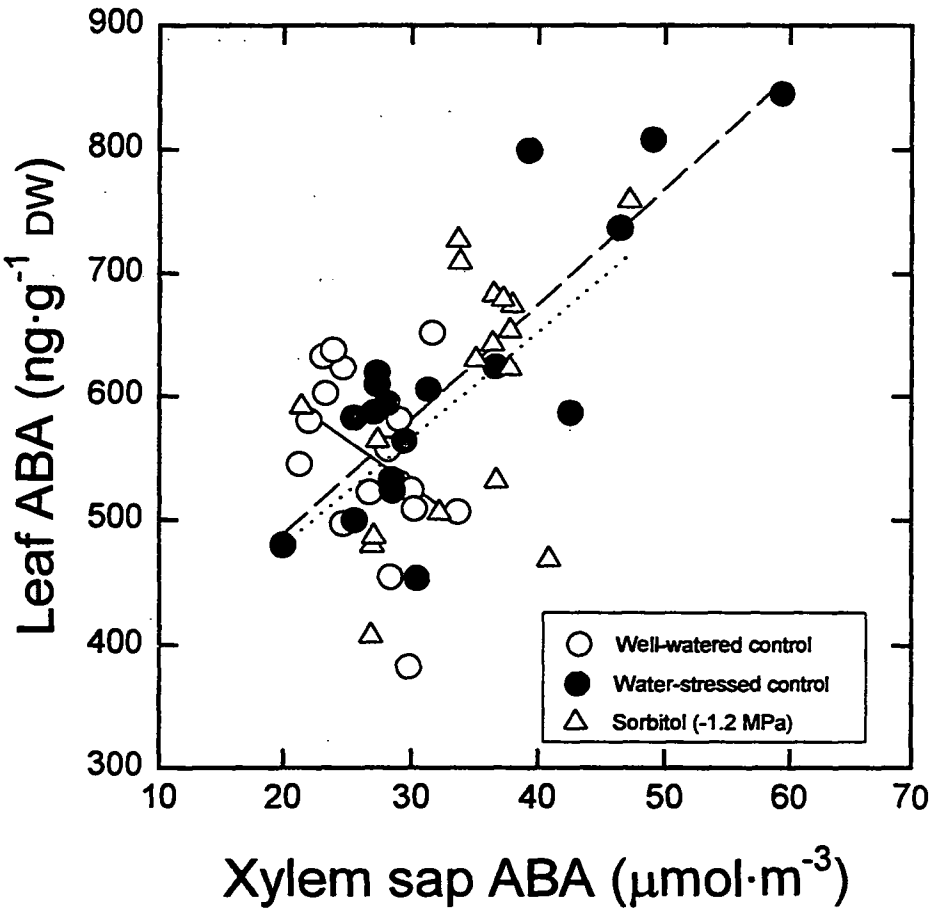

Figure 5-8. Relationship between leaf [ABA] and xylem sap [ABA] in apple (*Malus domestica* Borkh. 'Gala'/M26) for water-stressed controls (short-dashed) and sorbitol-treated plants (dotted-line). Regression equations: for water-stressed control, $Y = 303.07 + 9.30X$ ($R^2 = 0.78^{**}$, df = 17); for sorbitol-treated plants, $Y = 306.22 + 8.67X$ ($R^2 = 0.28^{*}$, df = 17).

analysis of linear relationship between g_s and the reciprocal of leaf [ABA] was significantly different between water-stressed controls and SIOS-treated plants (P = 0.004).

The [ABA] in the xylem sap, measured two hr after treatment, did not increase in response to SIOS treatment (Table 5-1). However, significant increases in xylem sap [ABA] occurred on Days 1 and 6 in SIOS-treated plants and water-stressed controls, respectively (Fig. 5-6). The level of xylem sap [ABA] in the SIOS-treated plants remained relatively constant until the end of experiment.

The relationship between g_s and xylem sap [ABA] was similar for both treatments (Fig. 5-7). With increasing xylem sap [ABA], in general, g_s decreased linearly to zero. Complete closure of stomata occurred at approximately 40 $\mu mol \cdot m^{-3}$ of xylem sap [ABA].

Leaf [ABA] increased linearly with increasing xylem sap [ABA] (Fig. 5-8). Correlation was tighter in the water-stressed controls (R^2 = 0.70) than in the SIOS-treated plants (R^2 = 0.28).

5.5 Discussion

Stomata show a dynamic response to the water transport capacity of roots and shoots, maintaining constant levels of leaf water status during adverse environmental conditions such as drought and osmotic stress (Sperry et al., 1993). Such a homeostatic stomatal mechanism is a kind of 'defense strategy' for plants to avoid leaf desiccation at the extreme. The SIOS treatment may enhance this defense strategy, regulating g_s (hence transpiration) by rapidly restricting hydraulic conductance within the plant. In this study, a rapid decrease in g_s in response to SIOS treatment was observed concurrently with

change in leaf water status, but not with either leaf or xylem sap [ABA]. This suggests that the effect of SIOS on g_s might result from hydraulic, rather than chemical, root signals. The rapid response of stomata also demonstrates that SIOS induces a rapid decrease in g_s by inhibiting water uptake by roots and perturbing the hydraulic pathway in the xylem (Tesky et al., 1983).

Hydraulic signals resulting from sudden changes in hydraulic conductivity (or transpiration) in the xylem provide a rapid means of communication in the soil-plant-atmosphere continuum because the velocity of the pressure wave can approach the speed of sound in water (Malone, 1993). Fuchs and Livingston (1996) also demonstrated a role for hydraulic signals in stomatal control. By pressurizing the roots of woody plants they reversed the effects of decreased g_s in dry soil. In recent experiments, described by Whitehead et al. (1996), a 7-year-old *Pinus radiata* tree was subjected to hydraulic shock by suddenly shading its lower foliage to support the theory that rapidly transmitted hydraulic signals resulted from sudden changes in transpiration. The velocity of the water flow (<0.2 m$\cdot$hr^{-1}) in *Pinus radiata* is far too slow for a chemical messenger to be transported within minutes from the roots to the stomata.

This hydraulic signal may trigger the release of ABA in the apoplast, which acts on the guard cells (Hartung and Slovik, 1991). Similarly, it was expected in the current study that SIOS developed a hydraulic signal that would in turn trigger ABA release in the apoplast, with total leaf [ABA] unchanged.

In the water-stressed control, however, there was a possible chemical root signal involved in stomatal control, because the considerable decrease in g_s was readily associated with significant increases in both leaf and xylem sap [ABA] when the soil

dried. This does not, however, rule out some interaction between hydraulic and chemical systems. Recent models of stomatal response to soil-drying incorporated such an interaction (Tardieu and Davies, 1993; Tardieu et al., 1993).

A relationship between xylem sap [ABA] and g_s was presented in a wide range of plant species: sycamore maple seedlings (Khalil and Grace, 1993), almond trees (Wartinger et al., 1990), and sunflower (Schurr et al., 1992). Our data also showed the close relationship of xylem sap ABA to g_s in both water-stressed controls and SIOS-treated apple plants. Initial stomatal closure in the SIOS-treated plants, however, may be more closely related to hydraulic signals than to chemical signals, as discussed above.

Xylem sap [ABA] doubled in water-stressed controls from the base level of 25 $\mu mol \cdot m^{-3}$ in the well-watered controls. This peaked [ABA] in the xylem sap (50 $\mu mol \cdot m^{-3}$) was considerably low compared to 400 $\mu mol \cdot m^{-3}$ in Douglas-fir (Fuchs and Livingston, 1996), 700 $\mu mol \cdot m^{-3}$ in white lupin (Correia and Pereira, 1995), and 3500-8000 $\mu mol \cdot m^{-3}$ in conifers (Jackson et al., 1995). Those values of xylem sap [ABA], which induced a substantial decline in g_s, were determined through an ABA feeding study. It did not consider the effect of leaf water status on stomatal conductance, because the test plants (without roots) were well maintained without stress in a series of ABA solutions. Therefore, those absolute values may not be representative of plants exposed to drying conditions, because the degree of stomatal closure caused by xylem-transported ABA from water-stressed roots may largely depend on the modulating effects of hydraulic signals (Tardieu et al., 1993). In other words, the stomatal sensitivity of the

plant to xylem sap [ABA] is a function of leaf or root water status. Stomatal control may depend on the interaction of chemical and hydraulic signals.

Interestingly, in this study leaf [ABA] failed to increase in response to SIOS, compared to the results in chapter 4 where leaf [ABA] increased significantly two hr after treatment (Guak, 1998). Pierce and Raschke (1980) showed that the capacity for accumulation of ABA in the leaf seemed to depend on leaf turgor. Turgor response to the SIOS markedly varied between the chapter 4 and current study. Following applications of the same strength of SIOS, changes in leaf turgor differed distinctly between the studies.

This could have been due to differing environmental conditions and plant materials used. In the current study, plants were moved from the lathhouse to the outdoors, conditioned 21 days before beginning the experiment (with an average 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ midday PAR. The previous study was conducted in the greenhouse (average 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ midday PAR) using plants moved from the lathhouse 21 days before beginning experiment. In that study, a significant increase in leaf [ABA] two hr after SIOS treatment was associated with the concurrent decline in Ψ_p (67%, from 1.06 to 0.35 MPa), whereas the outdoor-adapted plants showed a relatively slight decrease (25%, from 2.15 to 1.58 MPa). Leaf [ABA] did not change. In the water-stressed controls, however, both greenhouse- and outdoor-adapted plants showed equally substantial increases in leaf [ABA] when the soil dried and Ψ_p was reached near zero. This is accordance with Pierce and Raschke (1980), who observed that sensitivity of ABA production to changes in leaf water status progressively increased as turgor approached zero.

5.6 Conclusions

SIOS treatment induced a rapid decrease in the stomatal conductance (g_s) of potted young apple plants. This provided a means for maintaining relatively constant levels of leaf water status during a short-term drying period, compared to the water-stressed controls. Our data suggest that g_s in SIOS-treated plants may be regulated through a hydraulic signal when plants were subjected to water deficit stress rapidly induced by SIOS. The rapid decrease in g_s in response to SIOS, as measured two hr after treatment, corresponded to the rapid decrease in leaf water status (Ψ_w, Ψ_p, and RWC). The concentrations of ABA in the leaf and xylem sap was not involved in stomatal closure in response to SIOS treatment.

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CHAPTER 6

COMPARATIVE EFFECTS OF SORBITOL, POLYETHYLENE GLYCOL, AND SODIUM CHLORIDE ON WATER RELATIONS, GAS EXCHANGE, AND ABSCISIC ACID CONTENT OF THE LEAF AND XYLEM SAP IN APPLE (*Malus domestica* Borkh.) PLANTS

6.1 Abstract

The effects on water relations, gas exchange, and ABA content in the xylem sap and leaves of apple plants were compared between pure sorbitol and iso-osmotic GLK8924. In addition, sorbitol was compared with iso-osmotic polyethylene glycol (PEG 3500) and sodium chloride (NaCl), whose molecular weights are larger and smaller than sorbitol, respectively. The plants were monitored for 8 days.

Applying sorbitol as a soil drench resulted in significant decreases in stomatal conductance and photosynthesis in fully expanded leaves 24 hr after treatment. These reductions in gas exchange were related to significant decreases in leaf total water potential and turgor pressure. This treatment significantly increased [ABA] of the xylem sap and leaf after 1 and 3 days of treatment, respectively. The pattern of changes in the water relations, gas exchange, and ABA content was similar between sorbitol and GLK8924 treatments. Iso-osmotic PEG 3500 and NaCl also inhibited stomatal conductance and photosynthesis, with a greater reduction with PEG 3500. PEG 3500 treatment caused a greater decrease in turgor pressure than did sorbitol and NaCl applications through the comparatively greater decrease in water potentials and slower decrease in osmotic potentials. PEG 3500 treatment significantly increased [ABA] of the xylem sap and leaf 1 and 3 days after treatment, respectively, which was similar to

sorbitol treatment. In the NaCl treatment, however, the significant increase occurred 1 day after treatment for both xylem sap and leaf.

The effects of sorbitol on water relations, gas exchange, and ABA accumulation in the leaf and xylem were similar to those of iso-osmotic GLK8924. PEG 3500, with a relatively larger molecular weight, affected stomatal conductance, photosynthesis, and water relations more than did compounds with smaller molecular weights such as iso-osmotic sorbitol and NaCl.

6.2 Introduction

GLK8924 is the test name for sorbitol, produced by the Great Lakes Chemical Corporation (West Lafayette, IN). Since its purity was not known, we were interested in comparing the effects of pure sorbitol with those of iso-osmotic GLK8924, on water relations, gas exchange, and ABA accumulation. In addition, the effects of sorbitol were compared with those of iso-osmotic PEG 3500 and NaCl.

The physiological response of the plant to osmolytes often depends on the type of compound applied (Chazen et al., 1995). Plaut and Federman (1985), Termaat and Munns (1986), Zekri and Parsons (1990), and Perez-Alfocea et al. (1993) have reported on how water relations, gas exchange, and ABA accumulation respond to PEG and NaCl. PEG with a molecular weight greater than 3000 is non-toxic (Zwiazek and Blake, 1988), non-ionic, and not absorbed by plants (Mexel et al., 1975; Carpita et al., 1979). Thus applying PEG solutions to the roots can often subject higher plants to controlled negative

water potentials (e.g., Mexal et al., 1975; Zwiazek and Blake, 1988; Zekri and Parsons, 1990; Perez-Alfocea et al., 1993).

However, PEG, applied to the roots, has had consistently greater inhibitory effects on leaf elongation than has iso-osmotic NaCl in wheat, tomato, and citrus plants (Plaut and Federman, 1985; Termaat and Munns, 1986; Zekri and Parsons, 1990; Perez-Alfocea et al., 1993). Adverse effects of PEG on plant development have previously been related to inhibited oxygen availability to roots and to the presence of phytotoxic contaminants, such as heavy metal ions (e.g., Largerweff et al., 1961; Mexal et al., 1975; Plaut and Federmann, 1985). Chazen et al. (1995) recently reported other negative effects of PEG, such as greater inhibition of root water transport compared to NaCl. This leads to additional reductions in extensibility of the growing leaf cell walls, to additional ABA accumulation, and to greater inhibition of leaf growth. NaCl reduces the photosynthetic capacity of many species. This may be caused by direct stomatal closure or as a consequence of non-stomatal inhibition of photosynthesis (Downton, 1977; Gale et al., 1967).

The objective of this study was to compare the effects of pure sorbitol with iso-osmotic PEG, NaCl, and GLK8924 on water relations, gas exchange, and ABA accumulation in apple leaf and xylem sap. This study was conducted in conjunction with experiments in chapter 5, following the same methods. Two controls (well-watered and water-stressed) as well as the GLK8924 treatment (as described in chapter 5) were also included in the comparisons.

6.3 Material and Methods

6.3.1 Plant material and stress imposition

Bench-grafted 'Gala/M26' apple plants were potted in 3.8-liter plastic pots containing a 1:1:1:1 (by volume) mix of pumice, soil, coarse sand, and peat. They were grown in a lathhouse in Corvallis, Oregon. Plants were fertigated weekly with 200 ppm Peter's (20-20-20 with micronutrients) starting from 10 May 1995, and trained to single stems by removing axillary shoots. Trees (selected by size) were transferred to full sunlight on August 17 1995, 21 days before beginning the experiment, and arranged in a randomized complete block design (3 blocks with 15 single-replicate trees each). Until the experiment was initiated, the plants were grown on saucers (#8) to collect irrigation water and prevent water stress.

Plants were treated (as a soil drench; 400 ml per pot) with iso-osmotic sorbitol, PEG 3500, or NaCl solution, each at -1.2 MPa, at 11:00 a.m. on Day 0, and then not watered during an 8-day period. Iso-osmotic potentials of sorbitol, PEG 3500, and NaCl were determined from the relationship between osmolality and concentrations of each compound, as shown in Appendix E. Two controls (well-watered and water-stressed) as well as the iso-osmotic GLK8924 treatment (as described in chapter 5) were also included in the comparisons.

6.3.2 Soil and leaf water status and leaf gas exchange measurements

Gravimetric soil water content was determined at 1- to 3-day intervals, from the midsection of the pot, when wet and dry (after 72 hr at 85°C) weights of soil samples were used. (Fig. 6-1).

Three plants per treatment, one per block, were measured on each sampling date. Leaf gas exchange and water potential were measured near midday (11:00-14:00 hr) on clear days at 1- to 3-day intervals, starting one day before the first treatment. On Day 0, measurements were taken two hr after treatment. Quantum flux densities during measurement averaged 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Gas exchange (stomatal conductance and net photosynthesis) of the most recent, fully expanded leaves (upper 7 to 10) was measured with an open CIRAS-1 gas exchange system (PP Systems, Haverhill, MA).

After gas exchange measurements, the leaves were excised, placed in an insulated box with ice, and transported to the laboratory to determine leaf water relations, relative water content, RWC, and leaf [ABA]. The overall time from sample collection to water potential and RWC measurements was less than 10 min.

Leaf water potential was measured with a pressure chamber (PMS Instruments, Corvallis, OR). Independent studies have shown the pressure chamber to be more accurate than a psychrometer (Jensen et al., 1989). Afterwards, each leaf was divided in two; one half was wrapped in aluminum foil to later determine leaf [ABA]. The other halves were put in 1.5-ml eppendorf tubes for determining osmotic potential. All those samples were frozen in liquid nitrogen and stored at -80°C. For determining osmotic potential, these samples were smashed thoroughly for 1 min with a glass rod in the

eppendorf tubes. A few drops of sap were decanted into clean tubes and centrifuged at 12,000 g for 5 min to obtain a clear sap. The 8-µl sap was micropipetted onto a filter paper disk in a vapor pressure depression osmometer (Wescor 5100C, Wescor Inc.) and osmolality was determined (Pomper and Breen, 1996). Osmolality was then converted to osmotic potential (Ψ_s) (Unit: MPa at 25°C) by using 2.48 MPa per Osmol•kg^{-1} (i.e., Ψ_s = -Conc•RT at 25°C = Osmol x 2.48, where R is the gas constant and T is the temperature in Kelvin).

6.3.3 Leaf extract and xylem sap sample preparations

The freeze-dried leaf samples for ABA analysis were finely ground with a mortar and pestle, extracted overnight in ddH_2O at 4°C, and microcentrifuged at 13,000 g for 6 min.

Xylem sap was collected from belowground shank tissues (including the whole root system) at 1- to 3-day intervals. Plants were bare-rooted without additional water; excess water was blotted from the roots (mostly for well-watered controls). The shank was cut about 5 cm above the most basal adventitious roots. Approximately 3 cm of bark was removed from the top portion of the shank to prevent contamination of xylem and phloem sap. The root system was placed in the pressure chamber with the shank protruding. Pressures varied according to plant water status; 0.4 to 0.6 MPa and 1.0 to 2.5 MPa were applied to plants with little or no stress to mild or severe stress, respectively. Approximately 500 µl of sap was extracted from the former, whereas 200-300 µl of sap was collected from the latter. To prevent contamination from damaged surface cells the first few hundred µls were discarded (Wolf et al., 1990). The sap was

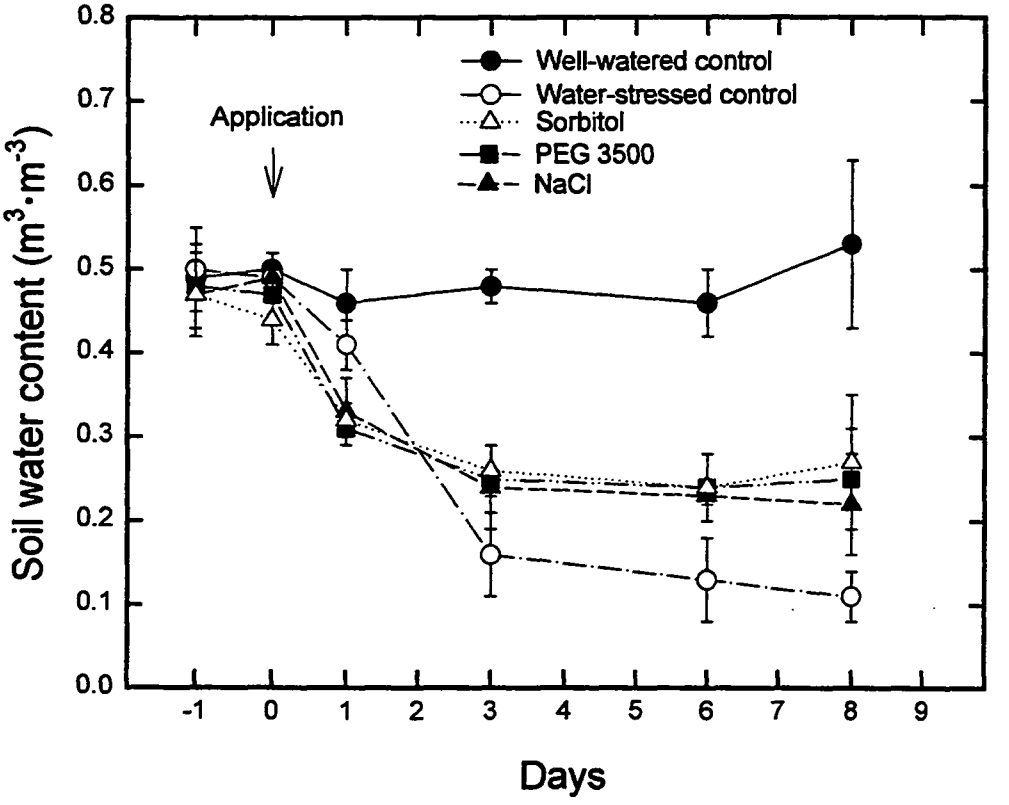

Figure 6-1. Time course of changes in gravimetric soil water content as drenched with sorbitol, PEG 3500, or NaCl solution, each at -1.2 MPa osmotic potential. After drench, water was withheld throughout the experiment. Values are means ± SE (n=3).

collected in 1.5 ml eppendorf vials in shade, then frozen in liquid nitrogen, and stored at -80°C. Xylem sap was collected from three plants per treatment for each sampling date. Just before use, the sap was thawed and microcentrifuged at 12,000 g for 2 min to obtain clean sap.

6.3.4 Determination of ABA content with radioimmunoassay (RIA)

The ABA content of the leaf extract and xylem sap was determined by RIA (Vernieri et al., 1989) using a monoclonal antibody to ABA from Sigma (Sigma Chemical Co. St. Louis, MO). Briefly, a PBS buffer (50 mM sodium phosphate and 100 mM NaCl, pH 7.0) was used. Approximately 2.5 $TBq.mmol^{-1}$ [G-^{3}H]-(RS)-Abscisic acid (Nycomed Amersham Place, Buckinghamshire, England, UK) in ethanol was diluted to 0.27 $\mu l.ml^{-1}$ in PBS containing 5.0 $mg.ml^{-1}$ bovine γ-globulin (Sigma), a co-precipitant with the antibody. The antiserum (Sigma), developed in rabbit with abscisic acid-HAS as the immunogen, was reconstituted and diluted in PBS containing the co-precipitant, per supplier's directions. Standard ABA solutions were prepared with (±) cis, trans-ABA (Sigma).

Solutions were added to 1.5 ml eppendorf assay vials in the following order: 50 µl of leaf extract or xylem sap, ABA standard or ddH_2O for B_{max} (100% binding of ABA tracer) determination, 100 µl [^{3}H-ABA] solution, and 50 µl antibody solution. Non-specific binding (B_{min}) was determined by omitting antibody from the assay mixture. The contents were vortexed gently and incubated for 30 min at 4°C. A saturated solution (200 µl) of ammonium sulfate (Grade 1, Sigma) was added to precipitate antibodies, and the mixture was then incubated at room temperature in the dark for 30 min. The precipitated

antibodies were pelleted for 6 min at 13,000 g in an eppendorf microcentrifuge. The supernatant was discarded and the pellet was washed by resuspending it completely in 400 µl of 50% saturated ammonium sulfate solution, centrifuging for 6 min and again discarding the supernatant. After removing as much supernatant as possible, the pellets were completely dissolved in 100 µl ddH_2O. A 1.2-ml cocktail solution (OPTI-FLUOR, Packard Instrument Co., Meriden, CT) was added, and the samples were counted twice in a liquid-scintillation counter (Packard Tri-Carb 1900CA, Packard Instrument Co.) for 10 min each time.

Generation of standard curves and the calculation of the ABA concentrations were described in detail in chapter 4 (Guak, 1998). Concentrations of ABA were calculated from the radioactivity present in the pellets. A series of six ABA standards (in twofold dilutions from 13000 pg to 130 pg per vial) was run in each batch of 28 samples for constructing a calibration curve. This was linearized by subtracting B_{min} and plotting logit-transformation of the corrected data against the Ln of unlabelled ABA present per vial, where Logit (B/B_{max}) = Ln $[(B/B_{max})/(1-B/B_{max})]$ and B is the corrected dpm bound in the presence of ABA standard. Sample ABA concentrations were interpolated from this line. The measured ABA never fell outside the range of the standard curve. The average correlation coefficient (R^2) of the standard curve was 0.98 (n = 8). The recovery of ABA for the sample was not determined. The validation of RIA, for use with crude apple xylem sap, was confirmed by a dilution/spike recovery test for non-specific interference, as described by Jones (1987). Validation of RIA for use with crude extract from the leaves of apple plants was reported in chapter 4 (Guak, 1998). A pooled sample of crude

xylem sap, undiluted or diluted to 50% and 25% with ddH_2O, was assayed in the presence of increasing amounts of synthetic (±) cis, trans-ABA standard (0.5, 2, and 4 pmol).

6.4 Results and Discussion

The effects on water relations, gas exchange, and ABA accumulation in the xylem sap and leaf, two hr after treatment, were similar for both sorbitol and iso-osmotic GLK8924 (Table 6-1). Sorbitol treatment significantly affected stomatal conductance, photosynthesis, water potentials, turgor potentials, and ABA concentrations in the xylem sap (but not in the leaf) compared to the well-watered controls. Time course measurements for sorbitol over an 8-day period were also similar to GLK8924 for soil water content (Fig. 6-1), photosynthesis (Fig. 6-2), and stomatal conductance, and water relations (Fig. 6-3). GLK8924 treatment data are presented in Figs. 5-1, 5-2, and 5-3 in chapter 5. These results indicate that GLK8924 probably did not contain any contaminants that would otherwise affect the results.

The PEG and NaCl treatments also significantly inhibited stomatal conductance and photosynthesis compared to the well-watered control over an 8-day period (Figs. 6-2 and 6-3). The inhibitory effects of PEG 3500 compared with iso-osmotic sorbitol and NaCl were most pronounced in stomatal conductance and photosynthesis when observed 1 day after treatment. PEG 3500 may have more strongly influenced gas exchange and water transport through roots than did NaCl or sorbitol, because of its higher viscosity (Chazen et al., 1995). The viscosity of aqueous solutions, such as PEG, increases rapidly with increasing molecular weight and concentration. For example, the reported viscosity of PEG 3500 solutions at -0.2 MPa was approximately 4 centipoise, compared with about

Table 6-1. Effects on water relations (water potential, osmotic potential, and turgor potential), gas exchange (stomatal conductance and photosynthesis), and ABA levels of the xylem sap and mature leaf of apple (*Malus domestica* Borkh. 'Gala'/M26) when roots are exposed to GLK8924 or sorbitol solution (-1.2 MPa) for two hr. Values are means ± (SE) (n=3).

Treatment	Potentials (MPa)			Stomatal conductance ($mmol \cdot m^{-2} \cdot s^{-1}$)	Photosynthesis ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Xylem sap [ABA] ($\mu mol \cdot m^{-3}$)	Leaf [ABA] ($ng \cdot g^{-1}$ DW)
	Water, Ψ_w	Osmotic, Ψ_s	Turgor, Ψ_P				
Well-watered control	-0.80 a[z]	-2.95 a	2.15 a	226.30 a	9.23 a	24.23 a	532.52 a
GLK8924 (-1.2 MPa)	-1.32 b	-2.90 a	1.58 b	101.60 b	6.22 b	27.64 a	551.73 a
Sorbitol (-1.2 MPa)	-1.30 b	-2.93 a	1.63 b	105.67 b	6.64 b	25.92 a	566.55 a

[z] Different letters within column indicate significant level at $P = 0.05$, means separated by t-tet.

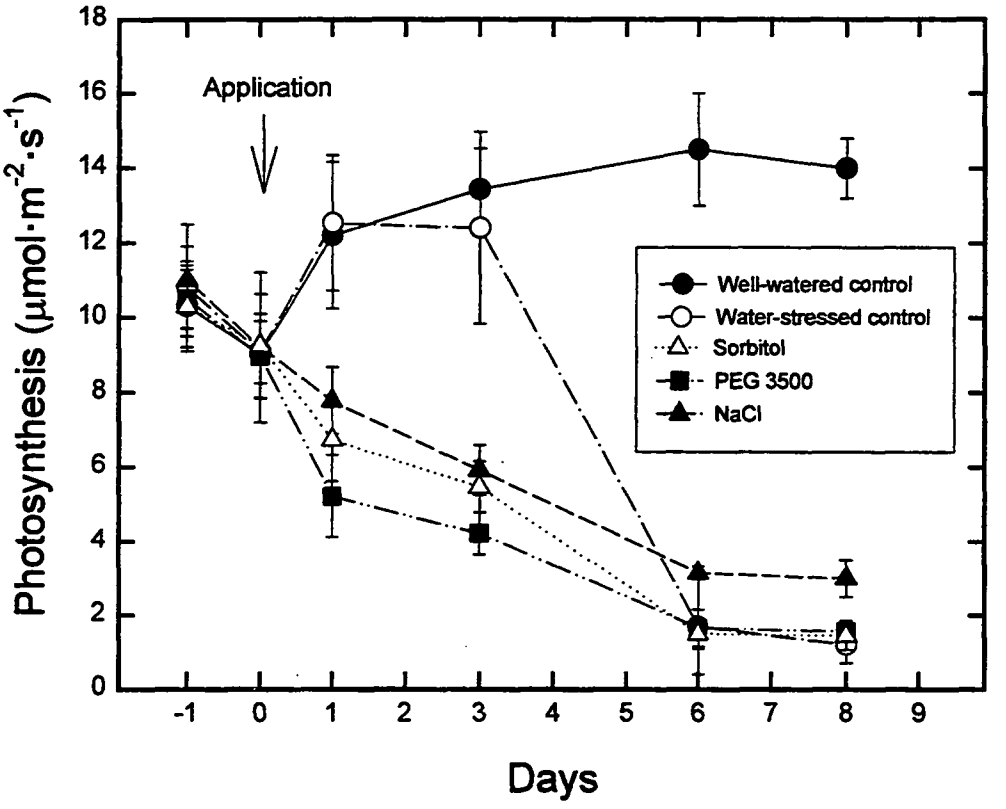

Figure 6-2. Time course of changes in the rate of photosynthesis in mature leaves of apple treated with either sorbitol, PEG 3500, or NaCl solution, each at -1.2 MPa osmotic potential. After treatment, water was withheld throughout the experiment. Values are means ± SE (n=3).

Figure 6-3. Time course of changes in (A) stomatal conductance, g_s, (B) water potential, Ψ_w, (C) osmotic potential, Ψ_s, and (D) turgor potential, Ψ_P in mature leaves of apple treated with either sorbitol, PEG 3500, or NaCl solution, each at -1.2 MPa osmotic potential. After treatment, water was withheld throughout the experiment. Values are means ± SE (n=3).

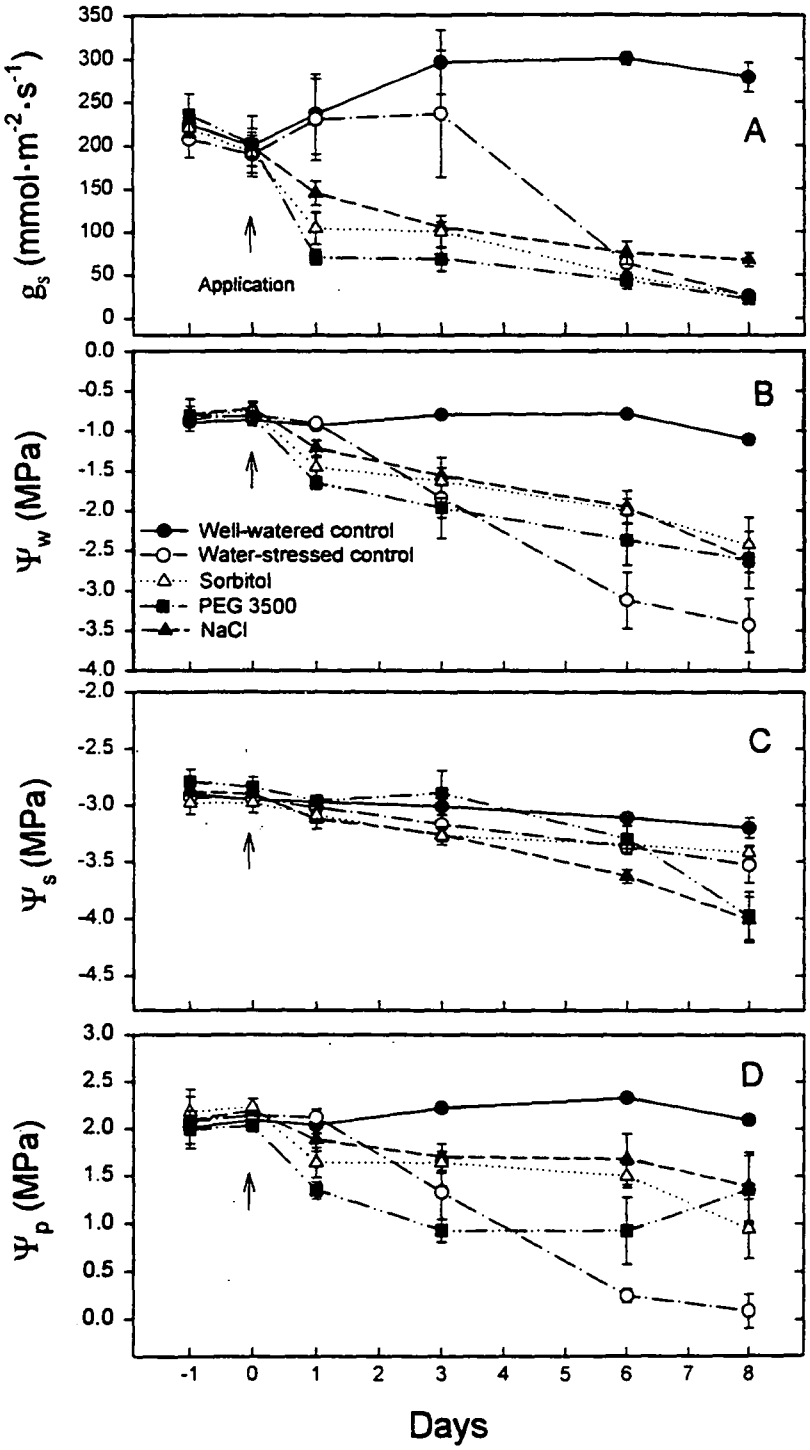
A
B
C
D
Application
Well-watered control
Water-stressed control
Sorbitol
PEG 3500
NaCl
g_s (mmol·m^{-2}·s^{-1})
Ψ_w (MPa)
Ψ_s (MPa)
Ψ_p (MPa)
Days

Figure 6-3

1 centipoise for lower molecular weight iso-osmotic NaCl (Michel and Kaufmann, 1973; Mexal et al., 1975).

The water potential of leaf tissues became more negative in the PEG treatment than in sorbitol and NaCl treatments (Fig. 6-3B). This might be explained by the different root water transport according to their viscosities. The osmotic potential in the PEG treatment, however, was not greatly affected until Day 6 and then decreased significantly by 0.62 MPa to a minimum of -4.00 MPa on Day 8. Osmotic potential in the NaCl treatment decreased gradually from -2.92 MPa on Day 0 to -4.02 MPa on Day 8 (Fig. 6-3C). Osmotic potential in the iso-osmotic sorbitol treatment decreased at slower rate than did NaCl, reaching a minimum of about -3.5 MPa on the final day. Consequently, turgor pressure showed a greater change in the PEG treatment than in sorbitol and NaCl treatments (Fig. 6-3D). Three days after treatment, for example, PEG 3500 treatment resulted in a turgor reduction of about 1.2 MPa compared to about 0.5 MPa in the sorbitol or NaCl treatment. Between Days 6 and 8, however, turgor potential in the PEG treatment recovered by about 0.5 MPa to the level of the NaCl treatment, because of the reduction in osmotic potential. However, photosynthesis and stomatal conductance levels did not recover (Figs. 6-2 and 6-3A).

Figure 6-4 shows that treatment of roots with sorbitol, PEG 3500, or NaCl each at -1.2 MPa osmotic potential induced increases in ABA levels of the fully expanded leaf tissues and xylem sap compared with the well-watered control. ABA fluctuations in NaCl-treated plants were quite different from those in water-stressed controls, sorbitol-, or PEG-treated plants. ABA in both leaf and xylem sap increased slightly but significantly 1 day after NaCl treatment, then decreased slightly by Day 3 to the level of

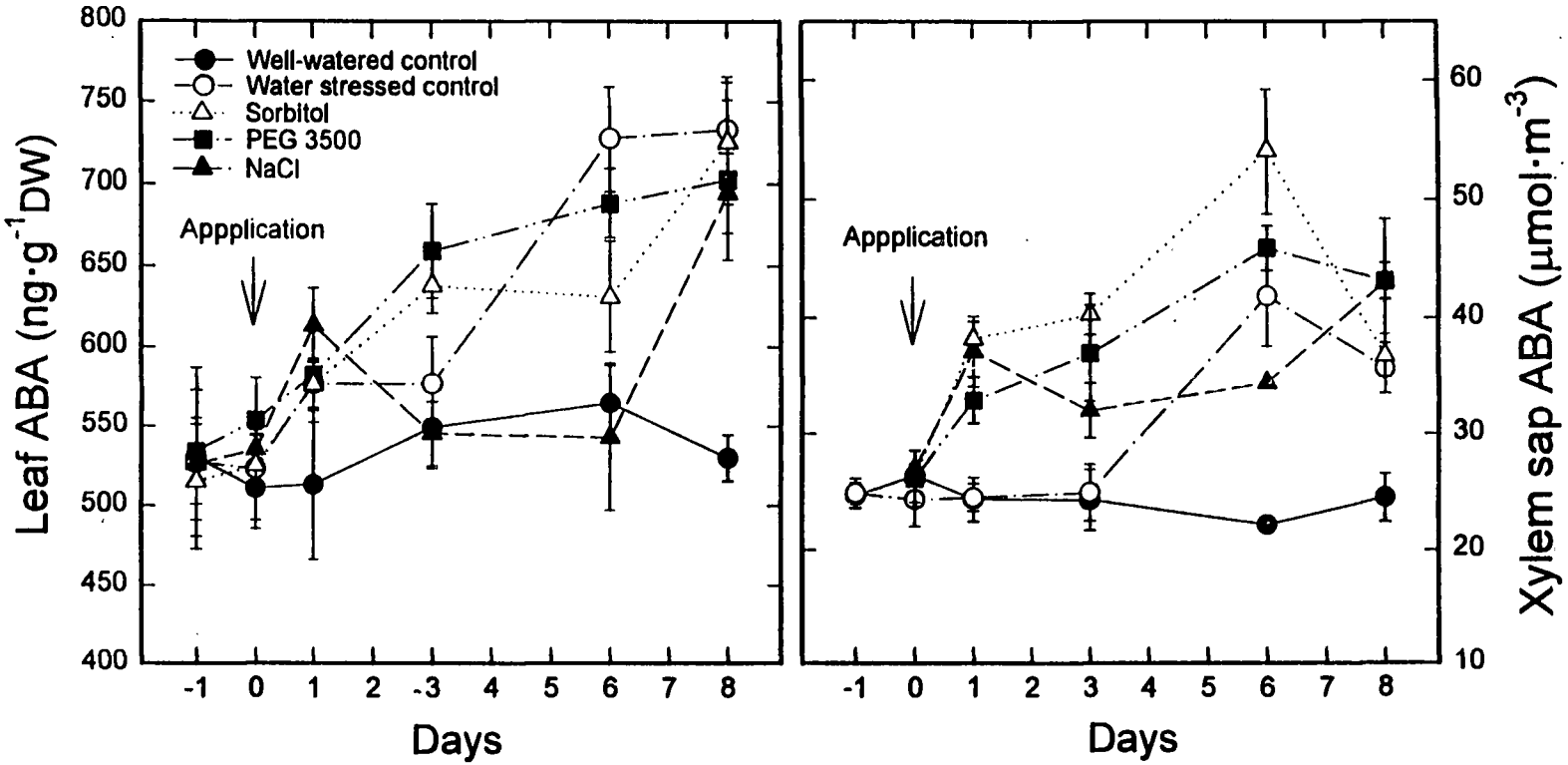

Figure 6-4. Time course of changes in the concentrations of leaf and xylem sap of apple treated with either sorbitol, PEG 3500, or NaCl, each at -1.2 MPa osmotic potential. After treatment, water was withheld throughout the experiment. Values are means ± SE (n=3).

well-watered controls for the leaf ABA. This level was still higher than in well-watered controls for the xylem sap ABA, and then increased again (peaked) between Days 6 and 8.

This phenomenon of the NaCl effect on ABA production was not well understood from this study. The slight increase in ABA on Day 1 in the NaCl-treated plants tended to disappear by Day 3. This might be because of turgor recovery through osmotic adjustment (Morgan, 1984), reductions in transpiration (Nobel, 1991; Khan et al., 1994), and greater root water uptake due to the relatively low NaCl viscosity (Chazen et al., 1995). The rapid increases in both xylem sap [ABA] and leaf [ABA] between Days 6 and 8 in the NaCl-treated plants seemed to be related to the lower soil water content than for that in sorbitol- or PEG 3500-treated plants.

In contrast, [ABA] of the xylem sap and leaf in the sorbitol- or PEG 3500-treated plants increased gradually to the maximum levels on Days 6 and 8, respectively. Xylem sap [ABA] tended to decrease (Fig. 6-4).

Figure 6-5 illustrates the close relationship between leaf [ABA] and xylem sap [ABA] in sorbitol, PEG 3500, and NaCl treatments.

ABA accumulates in the leaf tissues of plants responding to water deficits or salinity (Saab et al., 1990; Wolf et al., 1990). Although it was expected that ABA accumulations would be higher in the PEG-treated plants (because of viscosity-related reduced water uptake), this was not the case in our experiment.

We must still determine how root-applied osmoticum solutions signal the reduction in stomatal conductance when there is no significant change in bulk leaf [ABA] (e.g., on Day 1). Chazen and Neumann (1994) and Guak (1998; chapter 5) suggested the initial involvement of a hydraulic signal in stomatal control, i.e., increased xylem tension

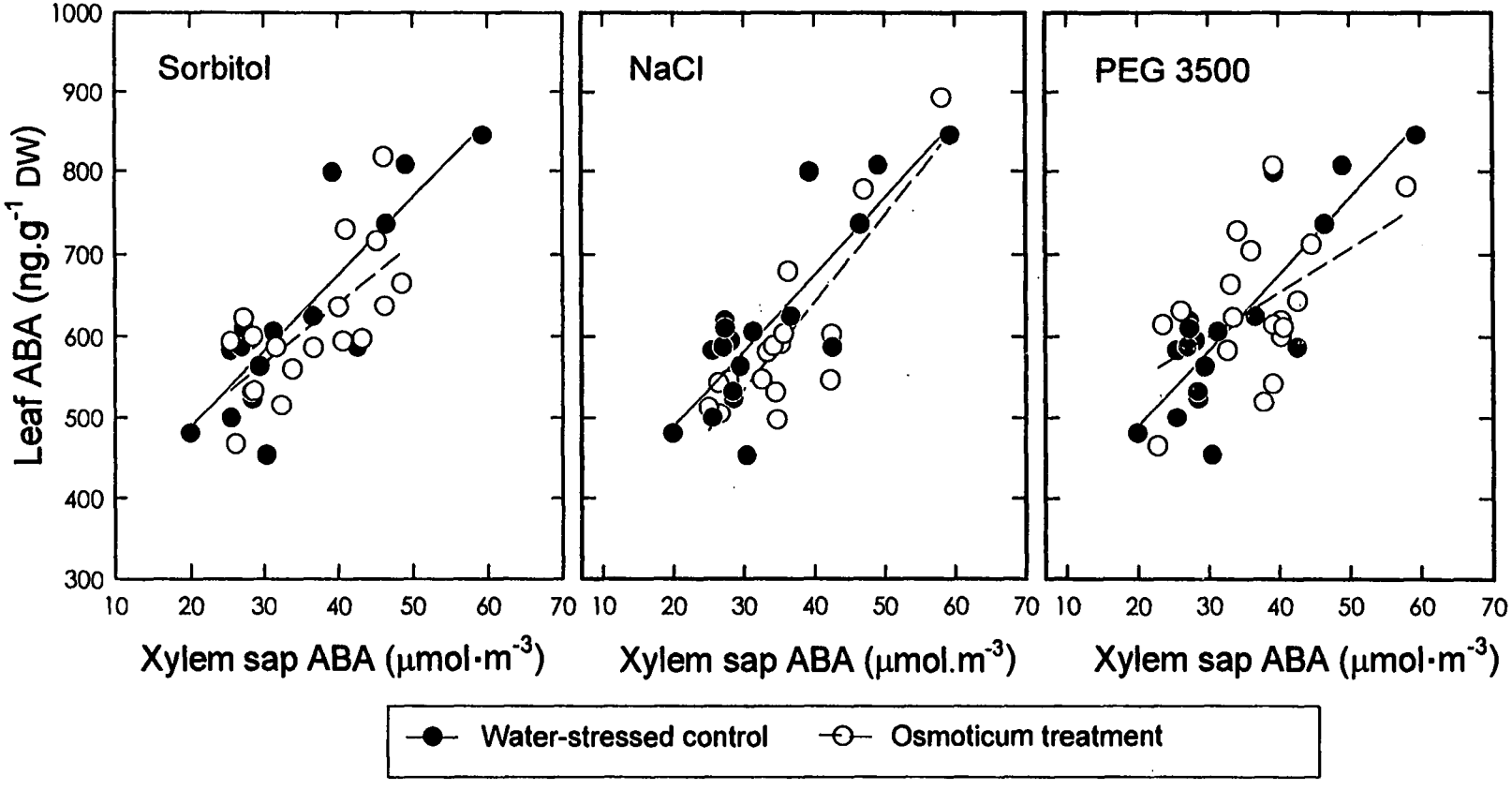

Figure 6-5. Relationship between leaf [ABA] and xylem sap [ABA] in apple treated with either sorbitol, PEG 3500, or NaCl each at -1.2 MPa osmotic potential. After treatment, water was withheld throughout the experiment. Regression equations: for water-stressed control, $Y = 303.07 + 9.30X$ ($R^2 = 0.70^{**}$); for sorbitol-treated plants, $Y = 349.76 + 7.26X$ ($R^2 = 0.47^{**}$); for PEG 3500-treated plants, $Y = 439.62 + 5.35X$ ($R^2 = 0.25^{*}$); and for NaCl-treated plants, $Y = 220.76 + 10.52X$ ($R^2 = 0.72^{**}$).

resulting from reduced root uptake and continuing evaporative loss from the shoots. Such tension probably leads to a transitory turgor decrease in epidermal cells, especially in stomatal guard cells (Zeiger, 1983). This might reduce stomatal conductance, although the turgor pressure of guard cells was not specifically determined in the current study. The increased [ABA] in leaves reported here for all treatments might subsequently contribute to maintaining a low level of stomatal conductance for several days.

6.5 Conclusions

The effects of pure sorbitol were similar to those of iso-osmotic GLK8924 for water relations, gas exchange, and [ABA] in the xylem sap and leaf. Iso-osmotic PEG 3500 and NaCl inhibited stomatal conductance and photosynthesis. PEG 3500 effects were greater than in either sorbitol or NaCl treatments. PEG 3500 also caused greater reduction in water potentials and turgor pressures than did sorbitol and NaCl. This may have been because the higher viscosity of PEG 3500 solutions inhibited root water transport more than did compounds, such as sorbitol and NaCl, which have lower molecular weights.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Water relations, gas exchange, and ABA concentrations of the leaf and xylem sap of bench-grafted container-grown apple (*Malus domestica* Borkh.) treated with sorbitol (-1.2 MPa) were studied. In addition, the effects of GLK8924 (the test name for sorbitol produced by Great Lakes Chemical Corp., West Lafayette, IN) on water relations, gas exchange, and ABA concentrations of the leaf and xylem sap were compared with those of iso-osmotic pure sorbitol to determine the purity. Effects of SIOS were similarly compared with those of other osmotica such as polyethylene glycol (PEG 3500) and sodium chloride (NaCl). General conclusions were drawn as follows:

1) Applying sorbitol as a soil drench caused the rapid reduction in stomatal conductance (as well as transpiration and photosynthesis) one hr after treatment. Consequently, the sorbitol-treated plants were significantly less stressed during 8 days of water stress period compared to the water-stressed control.

2) The initial decline in stomatal conductance was associated with turgor drop in the leaves. The sorbitol-induced osmotic stress (SIOS) treatment caused a rapid loss of turgor through its effect on water potential, while osmotic potential was rarely affected by treatment.

3) The leaves in SIOS-treated plants were less osmotically adjusted during the first day of treatment by 0.1 to 0.2 MPa than in the well-watered control. Toward the end of the drying period, an osmotic adjustment of about 0.5 MPa occurred in both water-

stressed controls and SIOS-treated plants. After rewatering, return of osmotic adjustment occurred faster in water stressed-control than in SIOS-treated plants.

4) Stomatal conductance was positively correlated with leaf water status, i.e., water potential, turgor pressure, and relative water content. The threshold values for inducing stomatal closure were about -2.0MPa and 85% for water potential and relative water content, respectively.

5) The after-effect of water stress on stomatal opening after rewatering was apparent in both water-stressed control and SIOS-treated plants, with that in the SIOS-treated plants being persisted much longer than in the water-stressed control.

6) The ABA production in the leaves in response to SIOS depended on the environmental conditions in which plants were adapted. In the greenhouse condition with an average midday PAR 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, leaf [ABA] increased significantly two hr after treatment, whereas it did not in the outdoor full-sunlight condition. The difference in the ABA production between two conditions would be associated with the concurrent differential decline of turgor. Turgor decreased by 67% (from 1.06 to 0.35 MPa) in the greenhouse-adapted plants, while it declined relatively slightly by 25% (from 2.15 to 1.58 MPa) in the outdoor-adapted plants.

7) Stomatal conductance significantly decreased two hr after SIOS treatment, similarly in both greenhouse and outdoor conditions.

8) The findings in 6) and 7) suggest that the effect of SIOS on stomatal conductance might result from hydraulic, rather than chemical, root signals.

9) The significant increase in [ABA] of the xylem sap and leaf in the SIOS-treated plants occurred 1 and 3 days after treatment, respectively, when the study was conducted in the outdoor condition. In water stressed-control, however, ABA levels of both xylem sap and leaf increased significantly on Day 6 (of water stress) when the soil dried and turgor pressure was reached near zero.

10) The peaked ABA levels in the leaves, regardless of treatments, decreased to the level of well-watered control about 3 days after rewatering. Stomatal conductance in water-stressed control was apparently recovering but never recovered in SIOS-treated plants, suggesting that the longer after-effect on stomatal opening in the SIOS-treated plants was not probably due to the delayed return of ABA level.

11) Leaf [ABA] and xylem sap [ABA] were negatively correlated with leaf water status (water potential, turgor pressure, and relative water content), observed commonly for water-stressed control and SIOS-treated plants.

12) Effects on water relations, gas exchange, and ABA concentrations of the leaf and xylem sap were similar between pure sorbitol and iso-osmotic GLK8924, indicating that GLK8924 probably did not contain any contaminants.

13) PEG 3500 reduced the rate of gas exchange (stomatal conductance and photosynthesis), water potentials, and turgor pressures more than did sorbitol and NaCl, which have lower molecular weights. This may have been because the higher viscosity of PEG 3500 solutions inhibited root water transport more than did compounds, such as sorbitol and NaCl.

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APPENDICES

APPENDIX A

RADIOIMMUNOASSAY FOR MEASURING ABSCISIC ACID IN THE CRUDE APPLE LEAF EXTRACT AND XYLEM SAP

A.1 Introduction

A radioimmunoassay (RIA) for abscisic acid (ABA) using the antibody has been developed with a great success. The antibody had a high selectivity for the free acid of (S)-cis, trans-ABA. Using the antibody, ABA could be assayed reliably in the RIA over a range from 100 to 4000 pg (0.4 to 15 pmol) ABA per assay vial (Quarrie et al., 1988). ABA was analyzed from crude aqueous extracts of wheat, maize, and lupin leaves without serious interference from other immunoreactive material. This successful use of RIA with crude extracts of leaves was improved by either measuring the distribution of immunoreactivity in crude extracts separated by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), or comparing the assay with physicochemical methods of analysis: Analysis of crude extracts by RIA and either, after TLC purification, by gas chromatography using an electron-capture detector or, after HPLC purification, by combined gas chromatography-mass spectrometry (GC-MS) gave very similar ABA concentrations in the initial leaf samples.

This appendix describes the RIA method we used for determining ABA in the crude extracts of leaves and the xylem sap in apple plants.

A.2 ABA Extraction

Freeze-dried apple leaves were ground with a mortar and pestle to pass through a 40 mesh screen. ABA was extracted by soaking 20 mg ground tissues for 24 hr in 1 ml ddH_2O in a 1.5 ml eppendorf tube on a vortex (shake level 3) at 4°C. Extracted solutions were microfuged at 13,000 g for 6 min. at room temperature and supernatants were transferred into clean vials.

Xylem sap was collected from belowground shank tissues (including the whole root system) at 1- to 3-day intervals. Plants were bare-rooted without additional water;

excess water was blotted from the roots (mostly for well-watered controls). The shank was cut about 5 cm above the most basal adventitious roots. Approximately 3 cm of bark was removed from the top portion of the shank to prevent contamination of xylem and phloem sap. The root system was placed in the pressure chamber with the shank protruding. Pressures varied according to plant water status; 0.4 to 0.6 MPa and 1.0 to 2.5 MPa were applied to plants with little or no stress to mild or severe stress, respectively. Approximately 500 µl of sap was extracted from the former, whereas 200-300 µl of sap was collected from the latter. To prevent contamination from damaged surface cells the first few hundred µls were discarded (Anderson and Brodbeck, 1989; Wolf et al., 1990). The sap was collected in 1.5 ml eppendorf vials in shade, then frozen in liquid nitrogen, and stored at -80 °C. Xylem sap was collected from three plants per treatment for each sampling date. Just before use, the sap was thawed and microcentrifuged at 12,000 g for 2 min to obtain clean sap.

A.3 Solution Preparations

PBS Buffer: It was made with 1.0 g Bovine γ globulin, 0.867 g NaCl and 1.38 g NaH_2PO_4 H_2O. Total volume was brought to 200 ml with ddH_2O at pH 7.0.

ABA-Antibody: The antiserum was developed by Sigma using abscisic acid-HSA as the immunogen. The product is diluted antiserum that had been lyophilized. The stock antiserum solution was made by dissolving one vial of lyophilized pellet with 1 ml PBS buffer and with gentle rotation. Five 200 µl aliquots of the stock antiserum in 1.5 ml eppendorf tubes were stored in a freezer at -20°C until use. When used, the aliquot of the reconstituted antiserum was further diluted with the 1.8 ml ddH_2O.

Tracer (^{3}H-ABA): The tracer was purchased from Amersham (Nycomed Amersham Place, Buckinghamshire, England, UK) with specific activity of 0.050 mCi. To prepare 200 tests, the 5.4 µl of ^{3}H-ABA solution (0.05 mCi) was added in 20 ml PBS buffer and stored in a refrigerator at 4°C. The final dpm of diluted ^{3}H-ABA solution was around 12600.

Standard ABA Solutions: Standard ABA solutions were prepared using 1mM ABA (±-cis, trans-ABA, Sigma) in methanol as described in Table B-1. In addition to the

standard ABA solutions, B_{min} (non-specific binding of ABA tracer) and B_{max} (100% binding of ABA tracer) values as well as pure tracer were needed. A total of 9 standards were run each time this procedure was done.

A.4 Radioimmunoassay (RIA) Procedures

1. Add 50 µl of each standard solution to respective 1.5 ml eppendorf tubes, add 50 µl of solution from freeze-dried samples (be careful not to get any solid material in the tube) or from xylem saps. Add 50 µl ddH_2O to B_{max} and B_{min}. Set aside three empty eppendorf tubes for tracer (^{3}H-ABA).
2. Add 100 µl tracer to each tube including three tubes set aside for tracer from step one.
3. Except B_{min} and tracer add 50 µl antibody solution to each tube. For B_{min} add 50 µl PBS buffer. Set aside tracer tubes, which will not be needed until step nine.

For the remaining tubes:

4. Close all tubes and vortex gently, and incubate for 30 min at 4°C.
5. Add 200 µl saturated $(NH_4)_2SO_4$ to all tubes except the tube only containing the tracer, vortex gently, and incubate for 30 min at room temperature.
6. Centrifuge at approximately 13,000 g for 6 min.
7. Discard supernatant and completely resuspend in 400 µl 50% saturated $(NH_4)_2SO_4$.
8. Centrifuge as above, discard as much of supernatant as possible (be careful not to remove any of the pellet on the pipette (in case pipette is used), and completely resuspend in 100 µl ddH_2O. Table A2 summarizes steps 1-8.
9. Add 1.2 ml scintillation cocktail (Packard Opti-fluor, Packard Instrument Co., Downers Grove, IL) to all tubes including tracer. Add scintillation cocktail to an empty tube to determine the background level of scintillation counter.
10. Vortex, incubate 60 min at room temperature, count twice for 10 min each on scintillation counter (Packard Tri-Carb 1900CA, Packard Instrument Co., Meriden, CT).

Regression of counts from ABA standard solutions 1-6 against Ln pg/vial from Table B1 was determined. This was linearized by subtracting B_{min} and plotting logit-

transformation of the corrected data against the Ln of unlabelled ABA present per vial, where logit (B/B_{max}) = Ln $[(B/B_{max})/(1-B/B_{max})]$ and B was the corrected dpm bound in the presence of ABA standard. Sample ABA concentrations were interpolated from this line. The mean R^2 for the linear regression was higher than 0.985 (n = 7). An example of standard curve is presented in Fig. 4-2 (Guak, 1998; chapter 4).

A.5 Validation of RIA: Dilution/Spike Recovery Test

To test for possible non-specific interference from substances in the crude extract of leaf tissues and crude xylem sap of apple plants, dilution/spike recovery test was conducted. This test consisted of adding a range of authentic ABA standards to the plant sample dilutions and checking that the amounts of ABA measured by RIA increased in proportion to the ABA amounts added (Jones, 1987; Pengelly, 1985; Zhang and Davies, 1990). Any deviation from the slope of ABA standards alone caused by the addition of tissue samples would indicate interference. Results of these tests were shown in Fig. 4-3 and 5-5 for crude leaf extract and crude xylem sap, respectively.

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Zhang, J. and W.J. Davies. 1990. Does ABA in the xylem control the rate of leaf growth in soil-dried maize and sunflower plants? J. Exp. Bot. 41:1125-1132.

Table A1. Preparations of ABA standard solutions and their final concentrations for ABA analysis using radioimmunoassay.

Solution Number	Dilution	ABA Solution	ddH_2O (ml)	Ln of pg/vial	pg/vial
1	1:1000	5 µl of 1 mM ABA (in methanol)	4.995	9.49	13226.80
2	1:5000	1 ml of solution 1	4.000	7.88	2643.87
3	1:10000	500 µl of solution 1	4.500	7.19	1326.10
4	1:20000	250 µl of solution 1	4.750	6.49	658.52
5	1:50000	100 µl of solution 1	4.900	5.58	265.07
6	1:100000	50 µl of solution 1	4.950	4.88	131.63

Table A2. Summary of ABA radioimmunoassay protocol.

Reagents	ABA Standards 1	2	3	4	5	6	Sample	B_{max}	B_{min}	Tracer
ABA std. Solution or sample (50 µl)	O	O	O	O	O	O	O	X	X	X
ddH_2O (50 µl)	X	X	X	X	X	X	X	O	O	X
Tracer ^{3}H-ABA (100 µl)	O	O	O	O	O	O	O	O	O	O
Antibody (50 µl)	O	O	O	O	O	O	O	O	X	X
PBS buffer (50µl)	X	X	X	X	X	X	X	X	X	O

Note: The O and X stand for addition and no addition of reagents, respectively.

APPENDIX B

THE INFLUENCE OF SORBITOL CONDITIONING BEFORE BARE ROOT HARVESTING ON TRANSPLANTING SUCCESS OF LAUREL (*Prunus laurocerasus* L.)

Transpiration, or water loss from various plant parts, is a natural process which can, under certain circumstances, result in either damage or death of plants. Water loss resulting from harvesting and bare-root transplanting may pose a problem to plant growth or survival in cases where the remaining roots may not be sufficient to compensate for the water lost in transpiration.

The objective of this study was to determine the feasibility of using sorbitol-induced osmotic stress (SIOS) in reducing water loss and increasing survival of transplanted broad leafed evergreen plants.

Sorbitol induces rapid stomatal closure when applied as a drench to the planting media. Dormant laurels (*Prunus laurocerasus* L. 'Otto Luken') [average 20 cm in height; propagated by cuttings in 8 cm x 15 cm polyethylene containers containing 1:1 mix of peat and bark (by volume)] were obtained from Oregon garden products (Forest Grove, OR). The media were drenched with either water, -0.75 MPa, or -1.5 MPa of sorbitol solution (50 ml per pot).

After 24 hr, plants were bare-rooted and subjected to either 0, 12, or 24 hr of desiccation stress on a bench in the greenhouse under day/night temperatures of 25°/18°C, with natural light and photoperiod. After each period of desiccation stress, plants were repotted individually in 3.8-liter pots containing 1:1:1:1 (by volume) mix of pumice, soil, coarse sand, and peat in the above greenhouse, watered, and placed on a greenhouse bench, and watered when necessary.

The midday stomatal conductance was measured one day after SIOS treatment with a Li-1600 steady-state porometer (LI-COR, Inc., Lincoln, NE). Shoot water potentials were measured with a pressure chamber (PMS Instruments, Corvallis, OR) immediately after each desiccation stress period. New shoot growth (dry mass) was determined in mid May three months after repotting.

Sorbitol application at both -0.75 MPa and -1.5 MPa caused significant decline in stomatal conductance 24 hr after treatment compared to the controls (Table B1). The midday shoot water potentials, measured after 12 and 24 hr of stress, were significantly higher in the SIOS-treated plants than in the water-stressed controls (Table B1). The -1.5 MPa treatment was more effective than -0.75 MPa in reducing water loss. The survival and growth of plants treated with SIOS at both drying periods was significantly greater than the water controls (Fig B2; Table B2).

This study suggests that preconditioning plants with SIOS prior to bare-rooting and transplanting may improve their survival and performance during establishment.

Table B1. Stomatal conductance of *Prunus laurocerasus*, determined 24 hr after treated with either water, -0.75 MPa, or -1.5 MPa of sorbitol solution as a drench to the planting media, and midday shoot water potentials were determined 0, 12, and 24 hr after desiccation stress.

Treatment	Stomatal conductance ($cm \cdot s^{-1}$)	Shoot water potential (MPa)		
		Periods of desiccation stress (hr)		
		0	12	24
Control	0.62 a[z]	-0.69 a	-2.22 c	-2.93 c
-0.75 MPa Sorbitol	0.31 b	-0.82 a	-1.87 b	-2.56 b
-1.50 MPa Sorbitol	0.11 c	-0.73 a	-1.49 a	-2.02 a

[z] Same letters within a column mean no significant difference in means (n=3), separated by Duncan's multiple range test at $P = 0.05$.

Table B2. New shoot growth of *Prunus laurocerasus* treated with either water, -0.75 MPa, or -1.5 MPa of sorbitol solution as a soil drench, bare-rooted 24 hr later, then exposed to either 0, 12, or 24 hr desiccation stress, and repotted and grown for 90 days in the well-watered condition. All procedures of the study was made in the greenhouse set at 25°/18°C (day/night) with natural light and photoperiod.

Treatment	New shoot growth (g dry mass)		
	Periods of desiccation stress (hr)		
	0	12	24
Control	27.5 a	8.2 c	1.5 c
-0.75 MPa Sorbitol	23.3 a	15.7 b	4.2 b
-1.50 MPa Sorbitol	25.7 a	19.2 a	14.2 a

[z] Same letters within a column mean no significant difference in means of n=10, separated by Duncan's multiple range test at $P = 0.05$.

Figure B1. Recovery of *Prunus laurocerasus* from desiccation stress. Pictures were taken 14 days after stressed-plants were repotted and watered. Plants treated with either water, -0.75 MPa, or -1.5 MPa of sorbitol solution were bare rooted 24 hr after treatment, then exposed to either 0, 12, or 24 hr desiccation stress, and repotted in the well-watered condition.

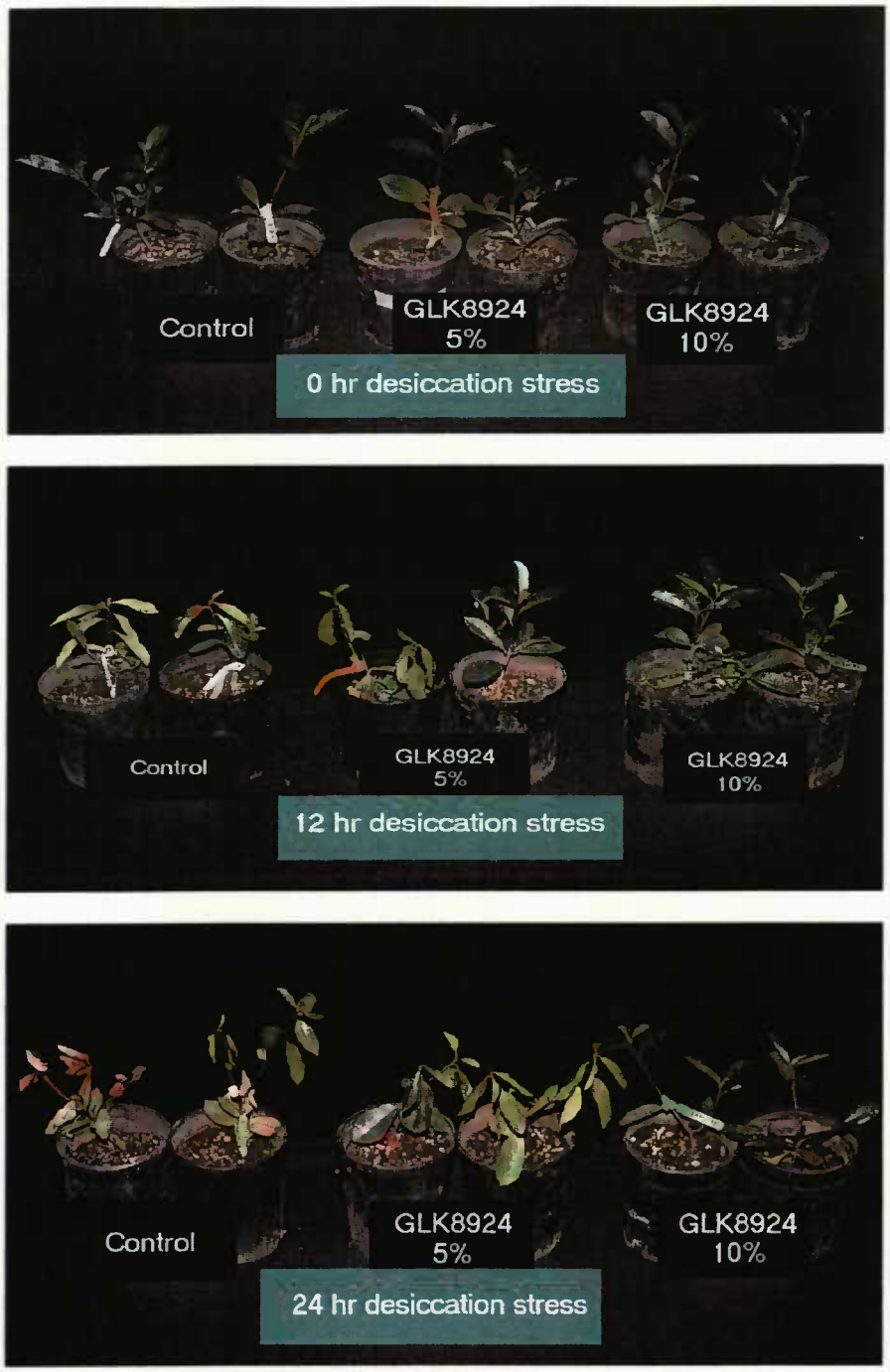
Control
GLK8924
5%
GLK8924
10%
0 hr desiccation stress
Control
GLK8924
5%
GLK8924
10%
12 hr desiccation stress
Control
GLK8924
5%
GLK8924
10%
24 hr desiccation stress

Figure B1

APPENDIX C

EFFECTS OF SORBITOL-INDUCED OSMOTIC STRESS ON TOLERANCE OF WHITE SPRUCE SEEDLINGS TO WITHSTAND DIRECT EXPOSURE TO DESICCATION STRESS BEFORE TRANSPLANTING

Water loss of nursery stock, during harvesting, storage, transplanting, and field establishment is a problem that can either reduce plant growth or cause plant mortality. The objective of this study was to determine whether sorbitol-induced osmotic stress (SIOS) treatment after harvesting could reduce water loss in white spruce (*Picea glauca* Voss.) seedlings during processing, storage, and transplanting. The effects of application timing (e.g., before or after storage) in combination with storage period on stomatal conductance, water potential, and evapotranspirational water loss were studied.

Two hundreds of one year-old white spruce (frost hardy northern stock) seedling plugs were obtained from Red Rock Research Station (B. C. Forest Service, Prince George, B. C. Canada) on 7 November, 1994. The seedlings were divided into two groups.

1) Group A (pre-storage treatment) seedlings were treated with either water, -1.1 MPa, or -1.5 MPa of sorbitol on 20 November, 1994, by soaking the root system and medium of ten plants each in plastic containers containing 1.5-liters of the above solutions for 19 hr in a greenhouse set at 25°/18°C (day/night) temperature and natural light. After soaking the media the excess solution was permitted to drain for 5 hr. After draining the treatments were further divided into 3 subgroups:

a. Subgroup 1 was placed immediately into 3.75 cm x 20 cm plugs in the greenhouse for desiccation resistance tests.

b. Subgroup 2 was wrapped with saran and placed in a cardboard box at -2°C for 60 days, removed from storage and transplanted into 3.75 cm x 20 cm plugs and placed in the greenhouse as subgroup 1.

c. Subgroup 3 was wrapped with saran, placed in a cardboard box at -2°C for 120 days, removed from storage and transplanted in 3.75 cm x 20 cm plugs and placed in the greenhouse as in subgroup 1 and 2.

2) Group B (post-storage treatment) seedlings were treated with water only as above on 20 November, 1994, wrapped with saran, placed in a cardboard box at -2°C for 60 and 120 days. Immediately after the storage period the seedlings were treated with either water, -1.1 MPa, or -1.5 MPa of sorbitol as above. After soaking the seedlings were placed into 3.75 cm x 20 cm plugs as above and placed in the greenhouse for desiccation resistance tests.

The desiccation stress treatment included placing the seedlings back into in 3.75 cm x 20 cm plugs (one seedling per plug) and then placing them in a 15 cm x 15 cm spacing in the greenhouse. Thereafter no water was added throughout the experiments except for the well-watered controls receiving daily watering. For recovery, the stressed-seedlings were transplanted in the same cells with potting media of 1:1:1:1 (by volume) mix of pumice, soil, coarse sand, and peat added, rehydrated by soaking in water for 3 min, and well-watered thereafter.

The relative weight change in the medium and seedling was determined during desiccation stress period. Shoot xylem water potential was determined with a pressure chamber (PMS Instruments, Corvallis, OR) 30 and 27 days after stress for 0 and 60 days storage treatment, respectively. The midday stomatal conductance of the terminal needle was measured 24 hr after treatment with a Li-1600 steady state porometer (LI-COR, Lincoln, NB). The values reported in Table C1 were the percent of water controls.

The SIOS treatment caused a rapid decrease in stomatal conductance 24 hr after treatment, regardless of application timing (i.e., before or after storage) and storage periods (Table C1). Consequently, shoot xylem water potentials after desiccation stress were significantly higher in the SIOS-treated seedlings than in the water-stressed controls (Table C2).

Relative weight change in the medium and seedling were significantly reduced by SIOS treatment (Fig. C1). In general, the rate of water loss was inversely proportional to the concentration of sorbitol, and seemed to be slower in the seedlings treated after storage than for those treated before storage, especially seen at the 120 day storage (Fig. C1c). After rewatering, most of the SIOS-treated seedlings recovered from the stress

treatment compared to the water-stressed controls (Fig. C2). Both concentrations of sorbitol were effective in reducing water loss and increased the tolerance of seedlings to desiccation stress before transplanting, and improved the survival of plug seedlings whether stored or not.

Table C1. Stomatal conductance of white spruce seedlings treated with either water, -1.1 MPa, or -1.5 MPa of sorbitol solution before or after seedlings were stored at -2°C. Measurements were made 24 h after treatment.

Treatment	Stomatal conductance (% of water control)				
	0 day storage	60 days storage		120 days storage	
		Pre[z]	Post	Pre	Post
Water control	100.0 a[y]	100.0 a	100.0 a	100.0 a	100.0 a
Sorbitol (-1.1 MPa)	20.2 b	31.5 b	23.8 b	36.5 b	25.4 b
Sorbitol (-1.5 MPa)	15.4 b	14.8 c	14.3 c	21.2 b	13.6 c

[z] Pre and post stand for treatment before and after seedlings were stored at -2°C, respectively.
[y] Same letters within a column mean no significant difference in means, separated by Duncan's multiple range test at $P = 0.05$.

Table C2. Shoot xylem water potential measured on the last day of desiccation periods in white spruce seedlings treated with either water, -1.1 MPa, or -1.5 MPa of sorbitol solution before or after storage at -2°C for 0 or 60 days. The seedlings of 0 and 60 day storage treatment were exposed to 30 days (from Nov. 29 to Dec. 20) and 27 days (from Jan. 26 to Feb. 22) of desiccation stress period, respectively.

Treatment	Shoot water potential (MPa)		
	Period of storage (days)		
	0	60	
		Pre[z]	Post
Well-watered control	-0.59 a[y]	-0.65 a	-0.69 a
Water-stressed control	-4.33 d	-4.07 d	-4.17 d
-1.1 MPa Sorbitol	-3.21 c	-3.35 c	-3.32 c
-1.5 MPa Sorbitol	-3.03 b	-3.12 b	-3.08 b

[z] 'Pre' and 'post' stand for treatment before and after seedlings were stored at -2°C, respectively.

[y] Same letters within a column mean no significant difference in means, separated by Duncan's multiple range test at $P = 0.05$.

Figure C1. Relative weight change in medium and white spruce seedling treated with either water, -1.1 MPa, or -1.5 MPa of sorbitol solution before or after cold storage at -2°C for 0 (A), 60 (B), and 120 (C) days, and then exposed to desiccation stress in the greenhouse for 30 days (from 21 Nov. to 20 Dec.), 27 days (from 26 Jan. to 22 Feb.), and 17 days (from 31 Mar. to 17 Apr.), for 0, 60, and 120 days storage treatment, respectively.

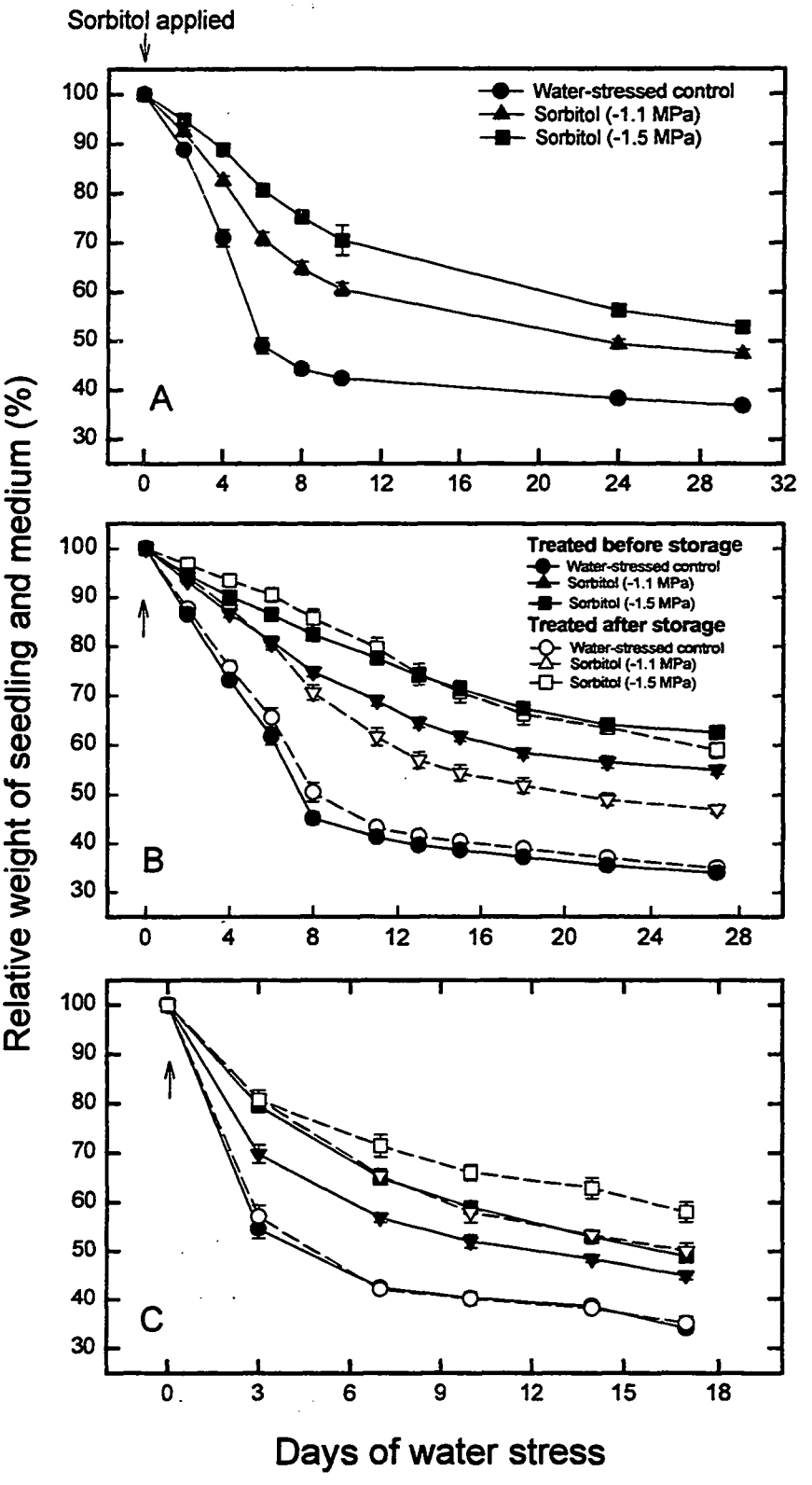
Sorbitol applied
Water-stressed control
Sorbitol (-1.1 MPa)
Sorbitol (-1.5 MPa)
A
Treated before storage
Water-stressed control
Sorbitol (-1.1 MPa)
Sorbitol (-1.5 MPa)
Treated after storage
Water-stressed control
Sorbitol (-1.1 MPa)
Sorbitol (-1.5 MPa)
B
C
Relative weight of seedling and medium (%)
Days of water stress

Figure C1

Figure C2. Recovery from desiccation stress of white spruce seedlings treated with either water, 7.5% (-1.1 MPa), or 10% (-1.5 MPa) of sorbitol solution, exposed to desiccation stress for 30 days, and then rewatered.

APPENDIX D

EFFECTS OF SORBITOL-INDUCED OSMOTIC STRESS ON STOMATAL CONDUCTANCE AND TRANSPIRATIONAL WATER LOSS IN VEGETABLES

The effects of sorbitol-induced osmotic stress (SIOS) on stomatal conductance and transpirational water loss were examined in tomato (*Lycopersicon esculentum* Mill. cv. 'Better Boy'), pepper (*Capsicum frutescens* L. cv. 'Jalapeno') and cucumber (*Trichosanthes cucumerina* L. cv. 'Flurry') plants. Seedling plugs, approximately 5 days after true leaf emergence, were obtained from Peoria Gardens Inc. (Peoria, OR), and transplanted into 5 x 7-cm square plastic pots on 15 April, 1993 with medium containing a 1:1:1:1 (by volume) mix of pumice, soil, coarse sand, and peat. Plant were grown in a greenhouse with 26°C day/20°C night temperature cycles and sodium vapor artificial light supplement (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, from 0500 to 2000 HR), fertigated weekly with 100 ppm Peter's (20-20-20 with micronutrients), and irrigated to prevent water stress when necessary. Pots were treated with four concentrations of sorbitol (0, -0.38 MPa, -0.75 MPa, or -1.5 MPa) at a rate of 50 ml per pot on 1 May at 10 a.m. Evaporational water loss from the medium was prevented by covering pots with sandwich bags after 4 hr of drainage. Water was withheld throughout the experiment.

Cumulative transpirational water loss was calculated as the sum of daily fresh weight difference of the medium and seedling. Stomatal conductance (g_s) was determined from fully expanded leaves with a Li-1600 steady state porometer (LI-COR, Inc., Lincoln, NB) at 11:00 a.m. daily. The xylem water potential was determined in pepper plants with the pressure chamber (PMS Instruments, Corvallis, OR) 7 days after treatment. Soil water content was determined in pepper plants 7 days after treatment by the following equation: relative soil water content (%) = (Final wt - Dry wt)/(Initial wt - Dry wt) x 100.

Sorbitol at -1.5 MPa was phytotoxic to all test plants and thus the results of -1.5 MPa treatment were not included in figures. The symptoms included leaf necrosis, epinasty and shoot dieback. The SIOS treatment was effective at -0.38 MPa and

-0.75 MPa in reducing g_s and transpirational water loss in all test plants, compared to water-stressed controls (Figs. D1, D2, and D3). The rate of decrease in g_s was proportional to the concentration.

Shoot xylem water potential and soil water content of pepper plants, determined 7 days after treatment, were significantly higher in the SIOS-treated plants than the water-stressed control (Fig. D4). This indicated that SIOS reduced transpirational water loss through stomata.

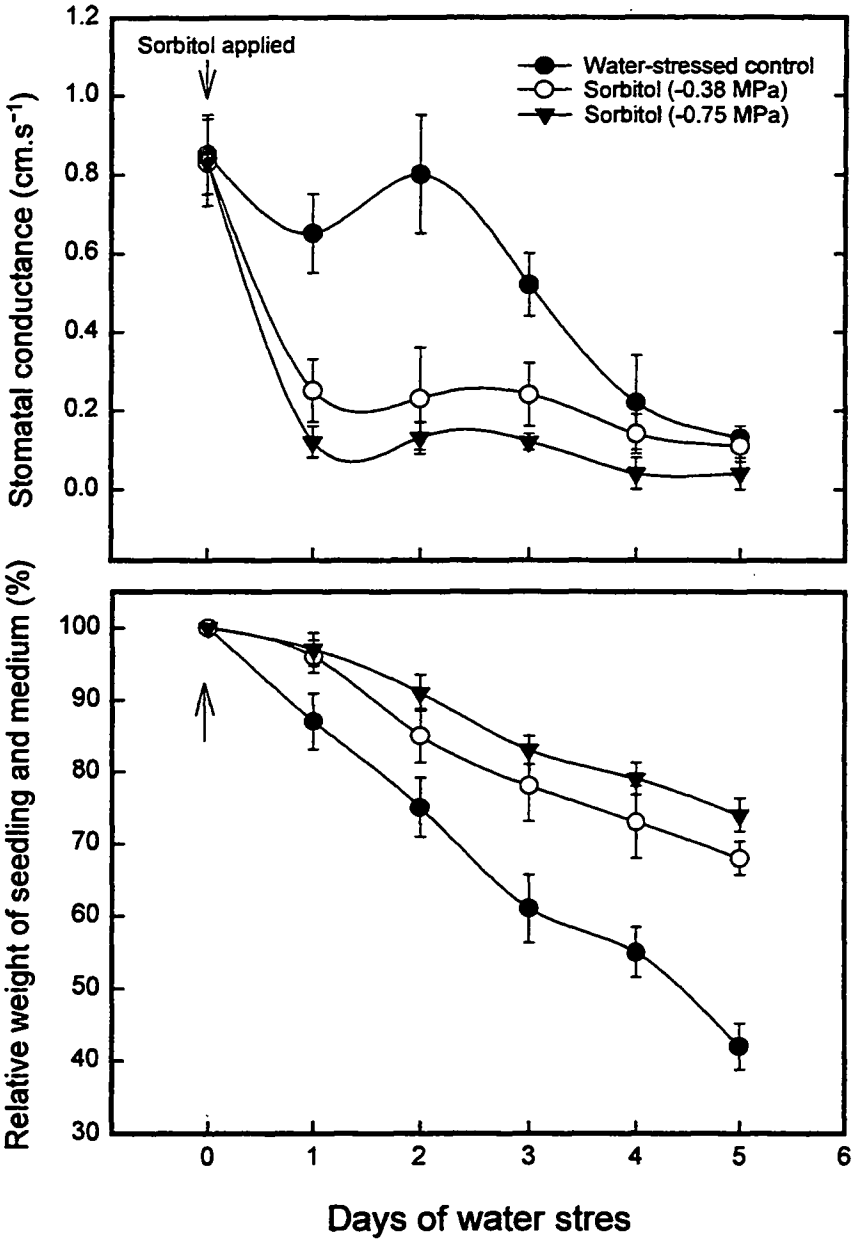

Figure D1. Effects of sorbitol-induced osmotic stress (SIOS) on stomatal conductance and cumulative transpirational water loss in tomato (*Lycopersicon esculentum* Mill.) plants.

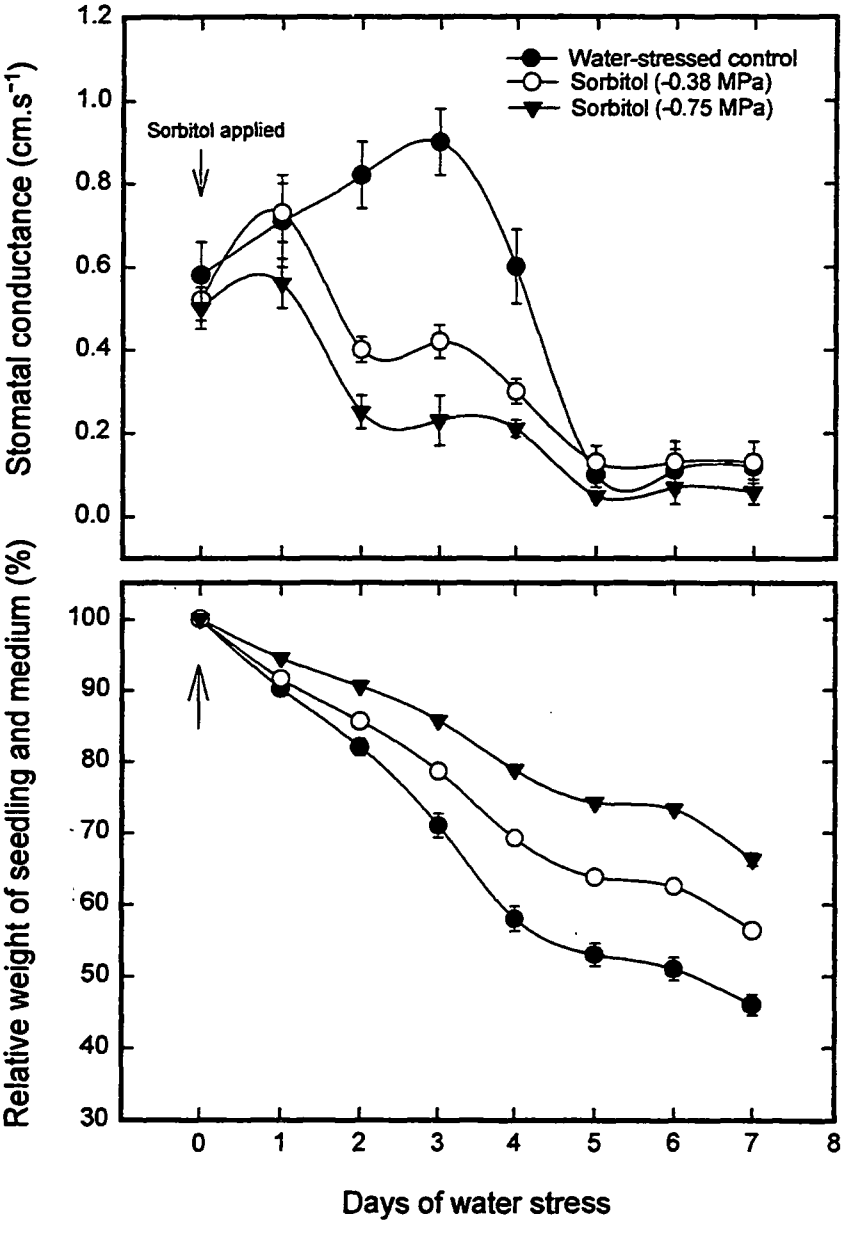

Figure D2. Effects of sorbitol-induced osmotic stress (SIOS) on stomatal conductance and cumulative transpirational water loss in pepper (*Capsicum frutescens* L.) plants.

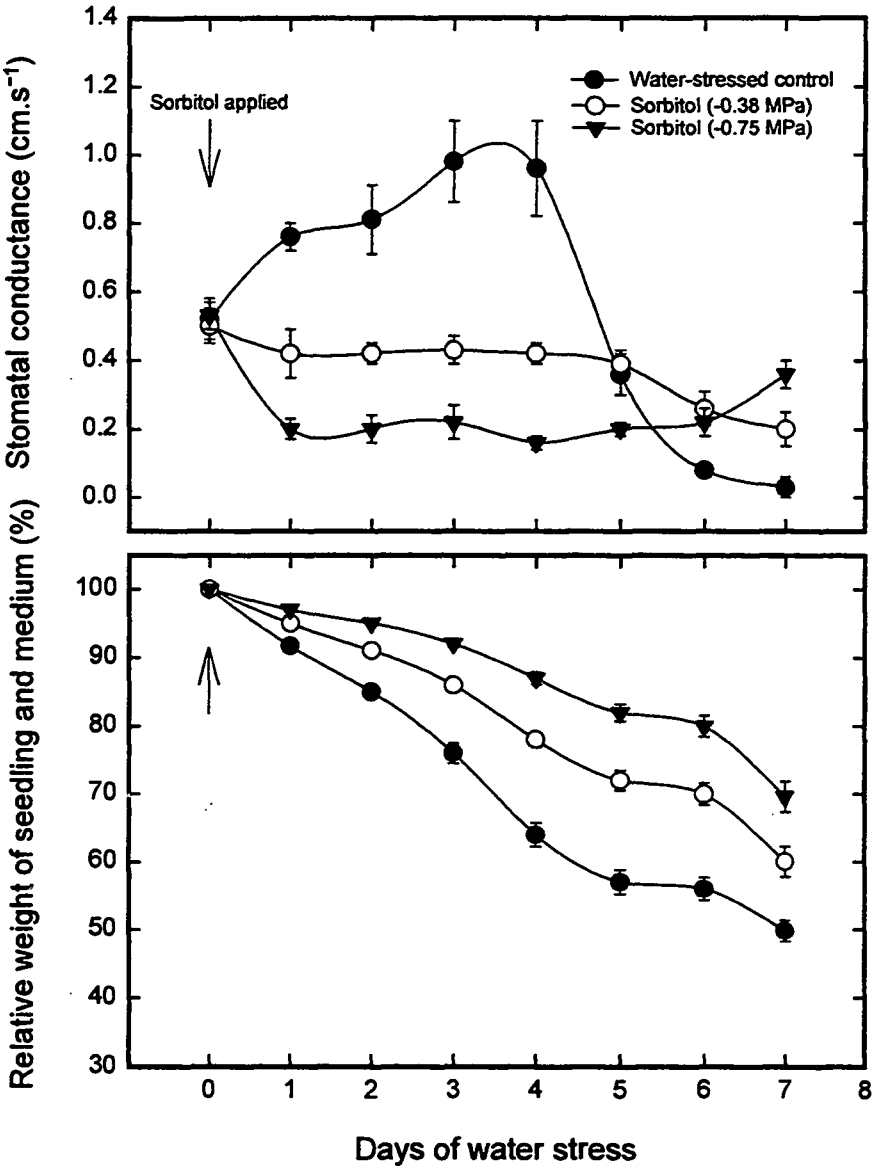

Figure D3. Effects of sorbitol-induced osmotic stress (SIOS) on stomatal conductance and cumulative transpirational water loss in cucumber (*Trichosanthes cucumerina* L.) plants.

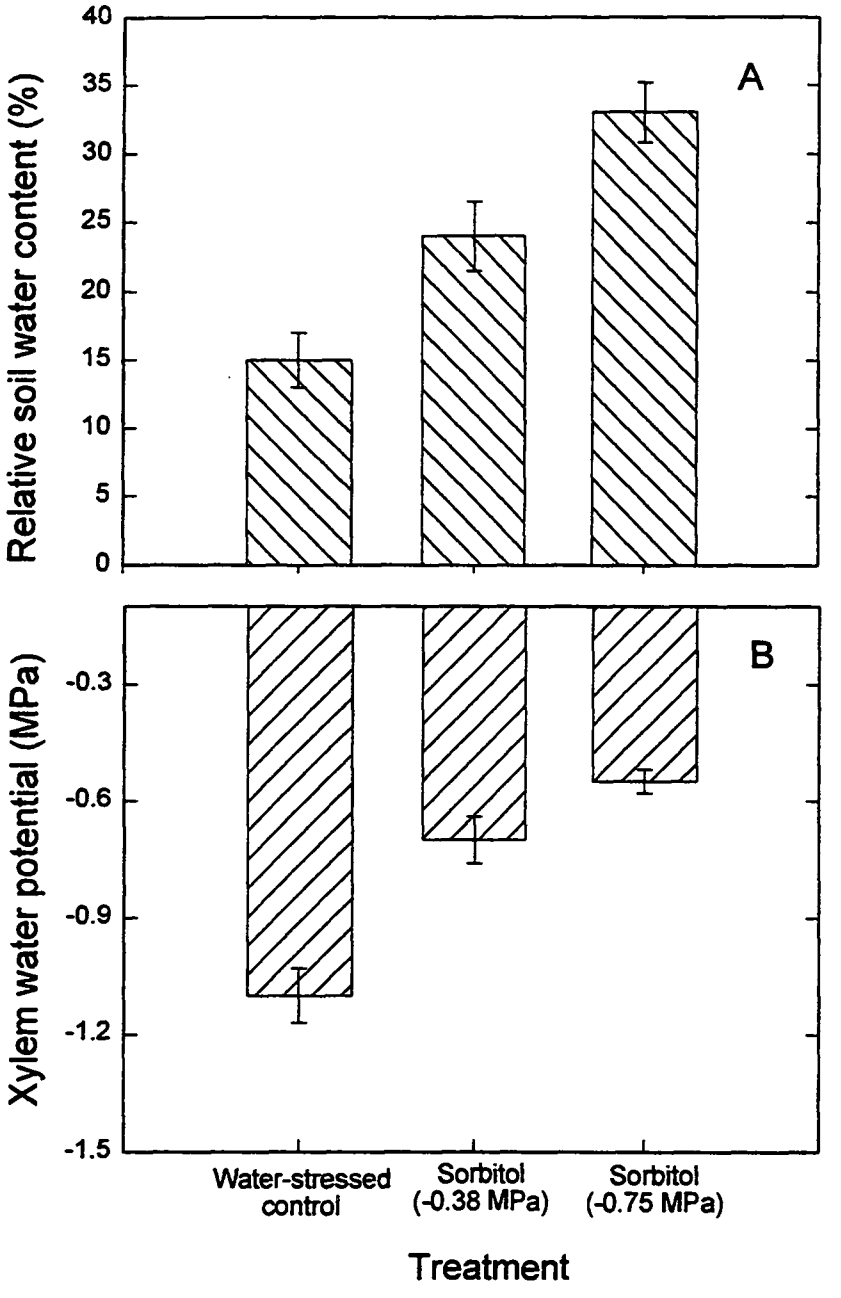

Figure D4. Relative soil water content and shoot water potential, determined 7 days after treatment from the pepper study.

APPENDIX E

RELATIONSHIP BETWEEN OSMOLALITY AND CONCENTRATION OF GLK8924, SORBITOL, PEG 3500, AND SODIUM CHLORIDE

The relationship of osmolality with concentration was determined for GLK8924 (Great Lakes Chemical Corp. in West Lafayette, IN), sorbitol, PEG 3500, and sodium chloride (NaCl). Solutions of GLK8924 and PEG 3500 were prepared on a percent basis, while those of sorbitol and NaCl on a mole basis. Compounds were dissolved in ddH_20 by being shaken at 120 rpm for 60 min at 22°C. The osmolality of solution was determined by Wescor Vapor Pressure Depression Osmometer (model 5100 C, Wescor Inc., Logan, UT), as described by Pomper and Breen (1996). Osmolalities were converted into osmotic potential (MPa) by multiplying with a factor of 2.48 (according to Van't Hoff's equation, i.e., $\Psi_s = -RT\Sigma C_j$, where R is the gas constant and T is the temperature in degree Kelvin and C_j is the summation of the concentrations of all the solutes in the solution.

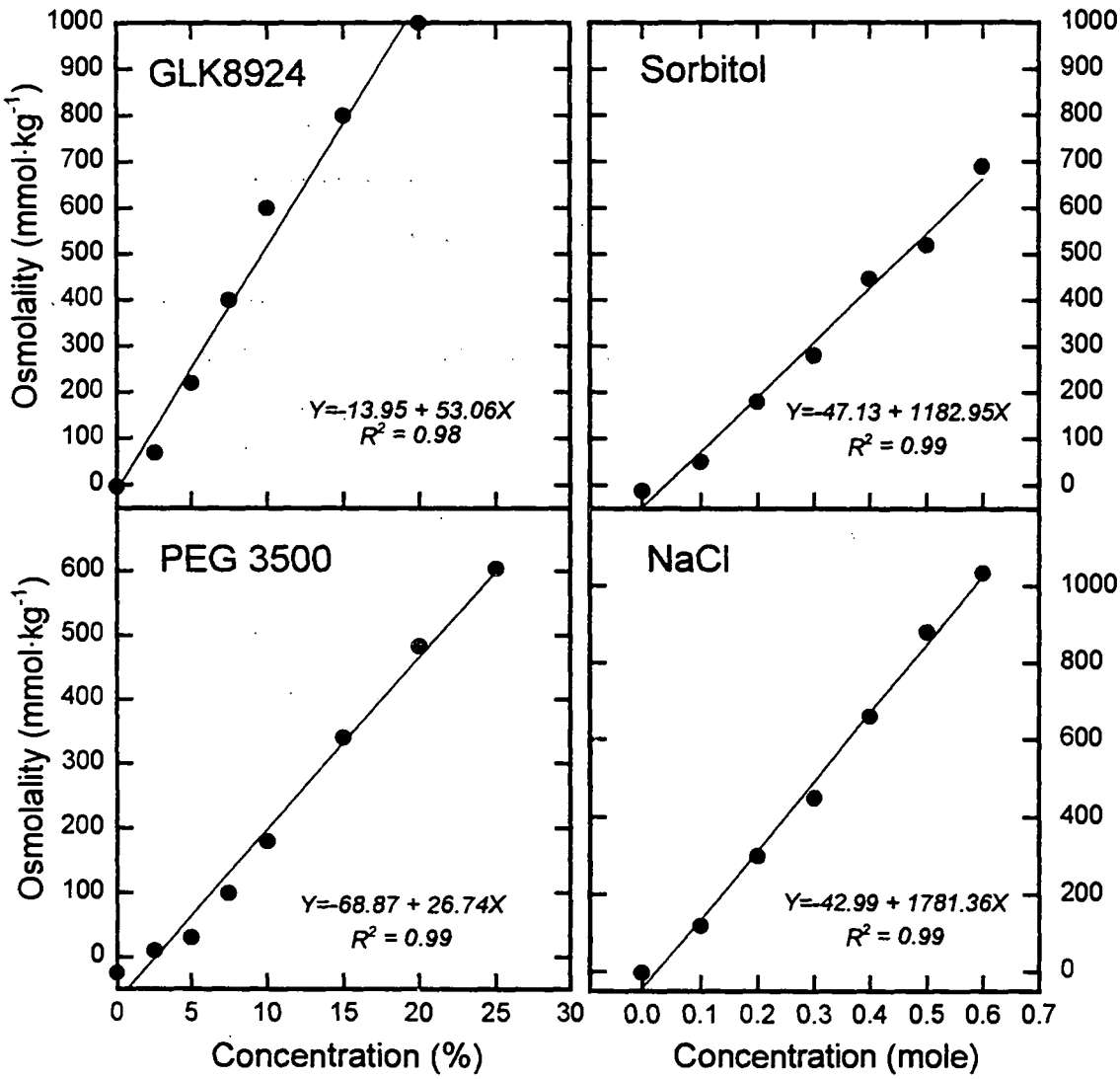

Figure E1. Relationship between concentration and osmolality of GLK8924, sorbitol, PEG 3500 and NaCl. Solutions of GLK8924 and PEG 3500 were prepared on a percent basis, while those of sorbitol and NaCl on a mole basis.

APPENDIX F

EFFECTS OF SORBITOL-INDUCED OSMOTIC STRESS ON DESICCATION RESISTANCE OF BARE ROOT BAGGED-ROSES

Five cultivars (Brandy, Blue Ribbon, Celebrate America, Grand Masterpiece, and Tournament of Roses) of dormant bare root bagged-roses (*Rosa hybrida* L.) were obtained from Bear Creek Gardens, Inc. (Medford, OR) on 14 March, 1994, and were stored at 4°C until the experiment. The size of bag was 12 cm diameter x 32 cm height and the medium was sawdust. Cane thickness was around 5 to 22 mm depending on cultivars and the number of canes per plant were three to six.

Plants were arranged on a bench in a randomized complete-block design with 10-single plant replicates. They were treated with either water or -1.2 MPa of sorbitol as a drench to the medium in the bag at the rate of 300 ml per bag on 24 March, 1994 in a greenhouse set at 26°/18°C (day/night) with natural light and photoperiod. One day after treatment, the medium was drained by making small holes on the bottom of the bag. Thereafter, water was withheld until 11 May, 1994, when shoots were wilted (or desiccated) and canes began to dieback. Stressed plants were rehydrated at the end of drying period by being soaked in water for 3 min and thereafter well watered. During drying period and after rewatering, measurements of relative weight change in the medium and plant, mean number of shoots per cane, percent shoot wilting, mean shoot growth, percent shoot recovery, and percent flower bud set were examined to test the hypothesis that sorbitol-induced osmotic stress (SIOS) by drench would increase desiccation resistance in bare root bagged-roses by retarding shoot growth (for the early stage of drying period compared to the water controls) and thus reducing water loss. The relationship between the extent of cane dieback and cane thickness was also determined for each cultivar. The extent of cane dieback was determined 25 days after rewatering by the visual injury scoring ranging from 0 = dead to 1 = healthy.

Water losses of the medium and plant were significantly reduced by SIOS treatment during a 48-day drying period, regardless of cultivars (Fig. F1). Mean number of shoots (longer than 1 cm) per cane, counted 17 days after treatment (and water stress),

was generally fewer in the SIOS-treated plants than in the water-stressed control (Fig. F2a). Averaged shoot growth per cane, measured 17 days after treatment (and water stress) by dividing the total length of the longest shoot per cane by the number of canes, was significantly reduced by SIOS treatment in most cultivars (Fig. F2b). Forty one days after water stress, more portions of shoots (longer than 4 cm) were wilted in the water-stressed control than in the SIOS-treated plants, seen in all cultivars except for Brandy (Fig. F3). Seven days later, however, shoots in the water-stressed control were significantly more stressed than in the SIOS-treated plants in all cultivars, with the percent wilting highest in 'Celebrate America' in both treatments (Fig. F3).

After rewatering, shoots were recovered more in the SIOS-treated plants than in the water-stressed controls, seen in all cultivars except for Tournament of Roses (Fig. F4a). In general, percent flower bud set, determined 25 days after rewatering, was higher in the SIOS-treated plants than in the water-stressed controls, especially in Brandy, Blue Ribbon, and Tournament of Roses (Fig. F4b). The relationship between the extent of cane dieback (observed 25 days after rewatering) and cane thickness showed that thicker canes were more resistant to desiccation stress, generally seen in all cultivars except for Celebrate America, regardless of treatment (Fig. F5). Furthermore, for certain thickness in 'Brandy' cultivar, canes were more healthy in the SIOS-treated plants than in the water-stressed control. Figure F6 shows shoot development 14 days after treatment and water stress (A), shoot wilting 40 days after treatment and water stress (B), shoot recovery 25 days after rewatering (C), and flowering 40 days after rewatering (D) for a cultivar 'Celebrate America'.

It is concluded that applying a hypertonic solution (about -1.2 MPa) of sorbitol as a drench to the medium significantly increased desiccation resistance of five cultivars of bagged-roses, by decreasing shoot growth and thus reducing evapotranspirational water loss compared to the water controls.

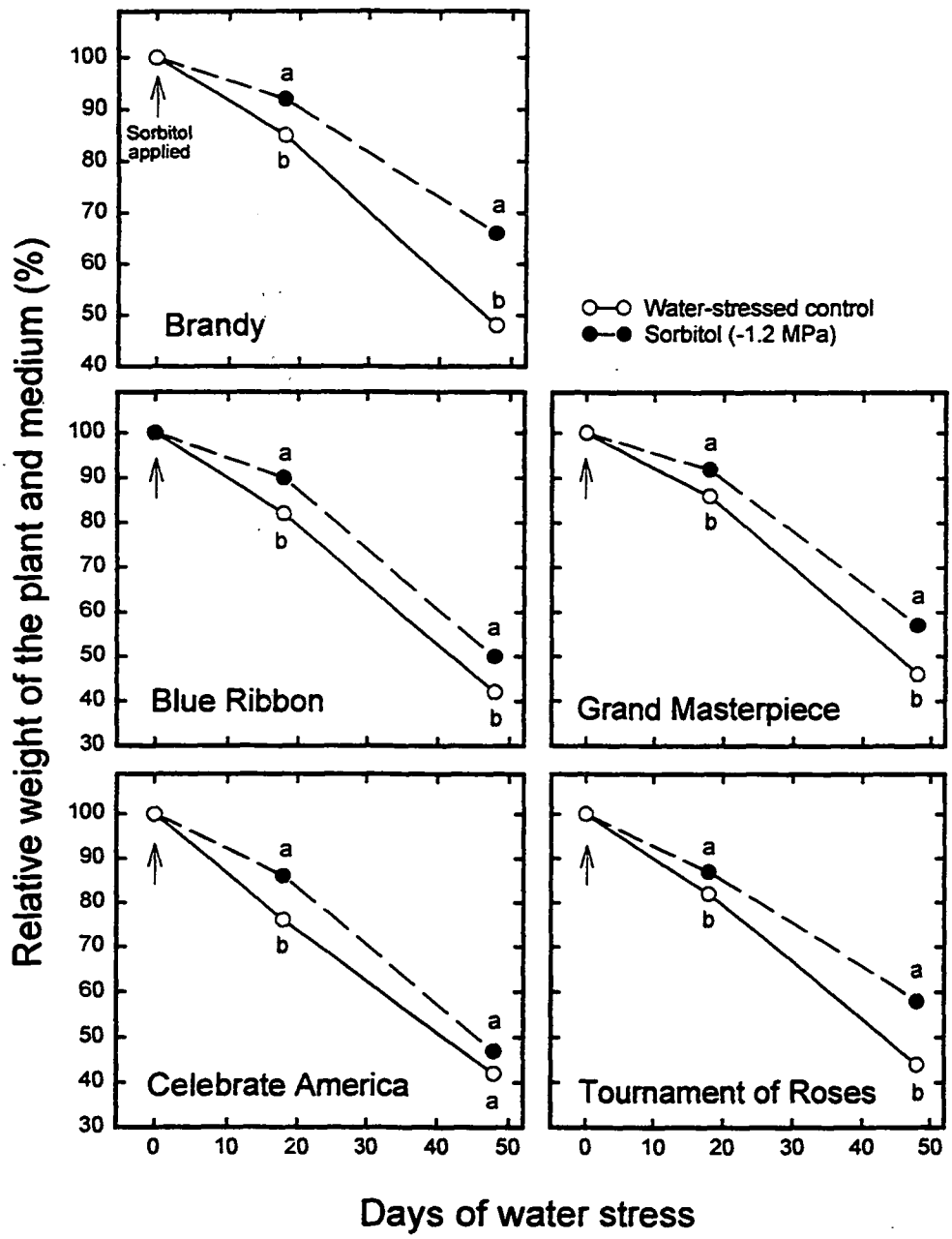

Figure F1. Changes in relative weight of the medium and plant of five bare root bagged-rose cultivars treated with either water or -1.2 MPa of sorbitol solution as a drench to the medium, with drying period. Relative weight (%) = (Initial weight-fresh weight)/Initial weight x 100.

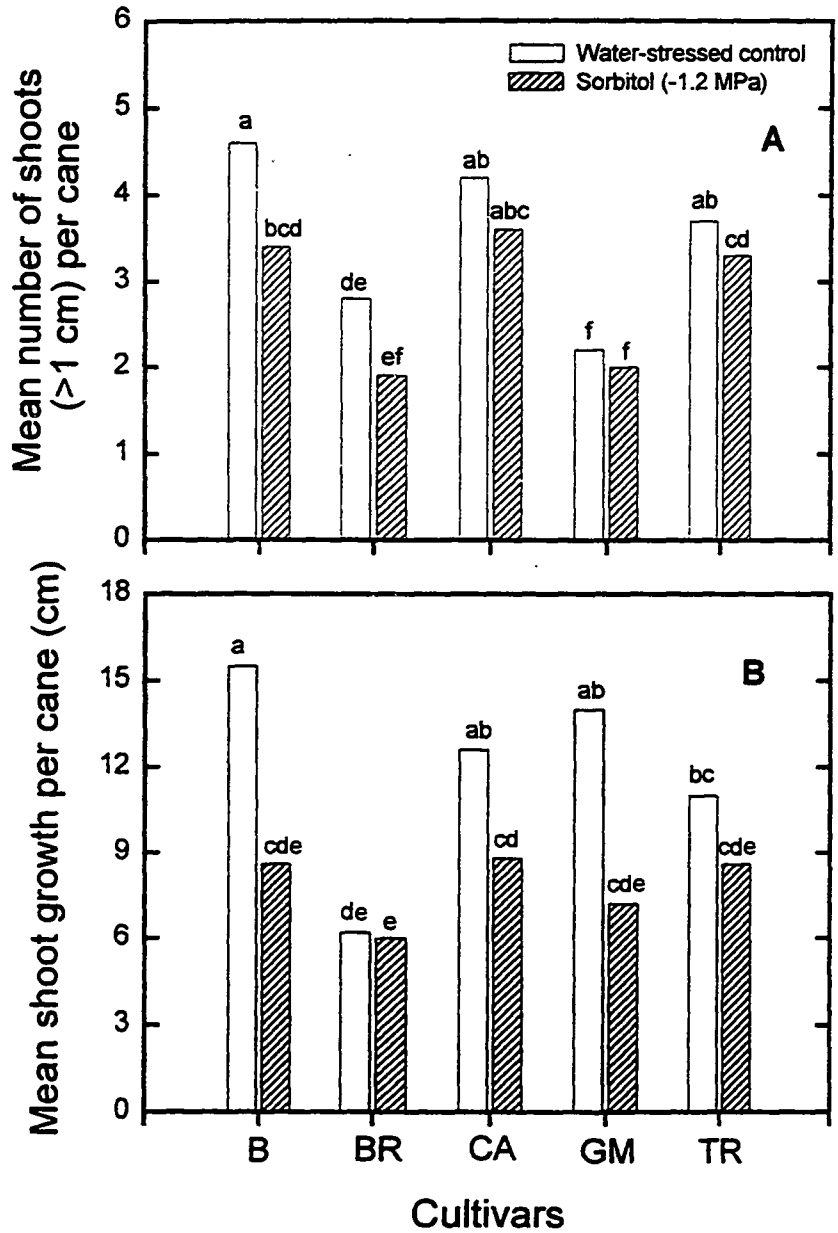

Figure F2. Mean number of shoots (longer than 1 cm) per cane (A) and mean shoot growth per cane (B), determined 17 days after treatment (and water stress) in five cultivars of bare root bagged-roses treated with either water or -1.2 MPa of sorbitol solution as a drench to the medium. Mean shoot growth per cane was determined by dividing the total length of the longest shoot per cane by the number of canes. Cultivar abbreviations: B = Brandy; BR = Blue Ribbon; CA = Celebrate America; GM = Grand Masterpiece; and TR = Tournament of Roses.

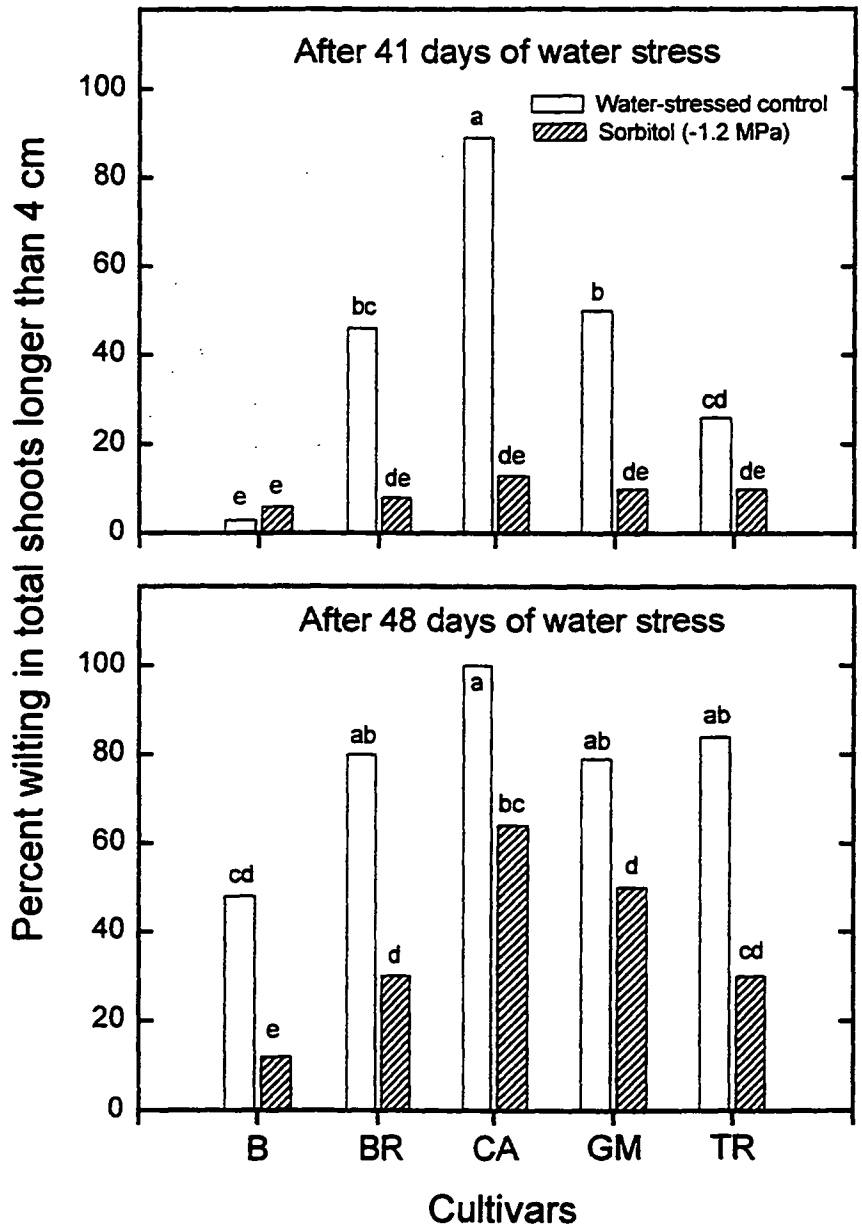

Figure F3. Percent wilting in total shoots longer than 4 cm, measured 41 and 48 days after water stress, in five cultivars of bare root bagged-roses treated with either water or -1.2 MPa of sorbitol solution as a drench to the medium. For cultivar abbreviations, see Figure F2.

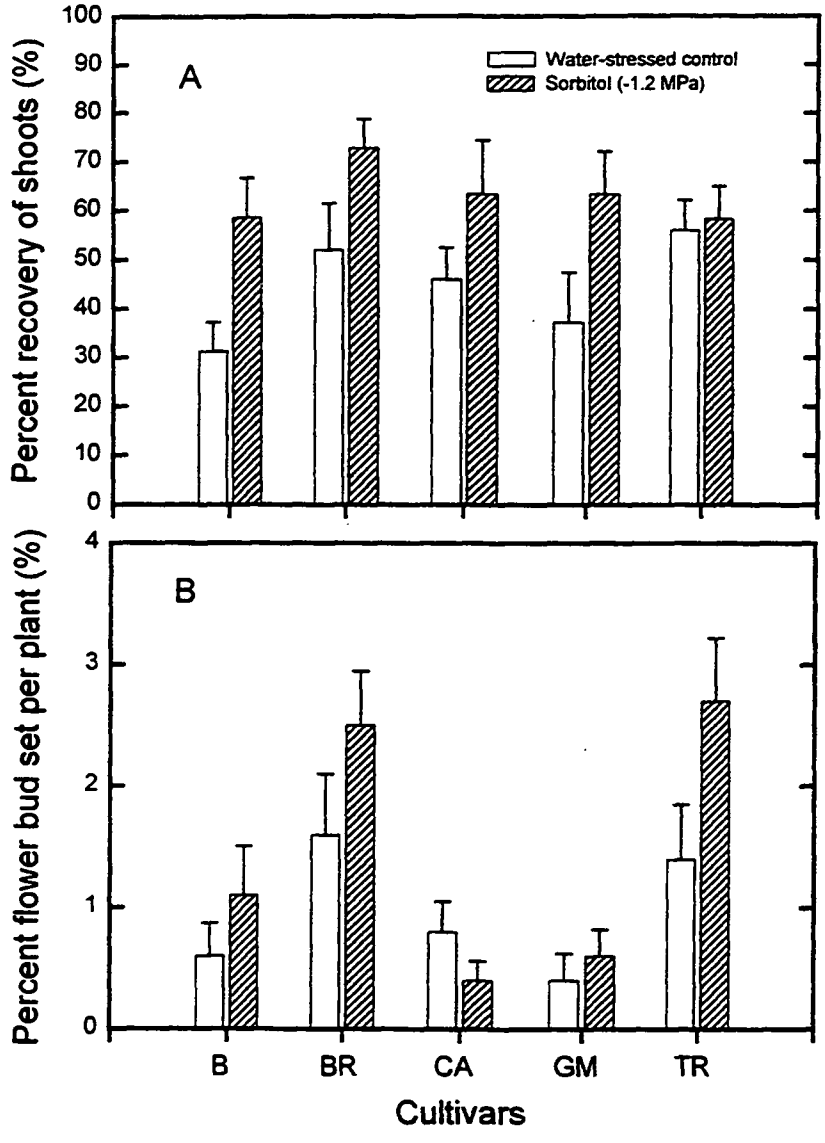

Figure F4. Percent shoot recovery measured 7 days after rewatering (A) and percent flower bud set measured 25 days after rewatering (B) in five cultivars of bare root bagged-roses treated with either water or -1.2 MPa of sorbitol solution as a drench to the medium. For cultivar abbreviations, see Figure F2.

Figure F5. Relationship between cane thickness and extent of cane damage determined 25 days after rewatering in five cultivars of bare root bagged-roses treated with either water or -1.2 MPa of sorbitol solution as a drench to the medium.

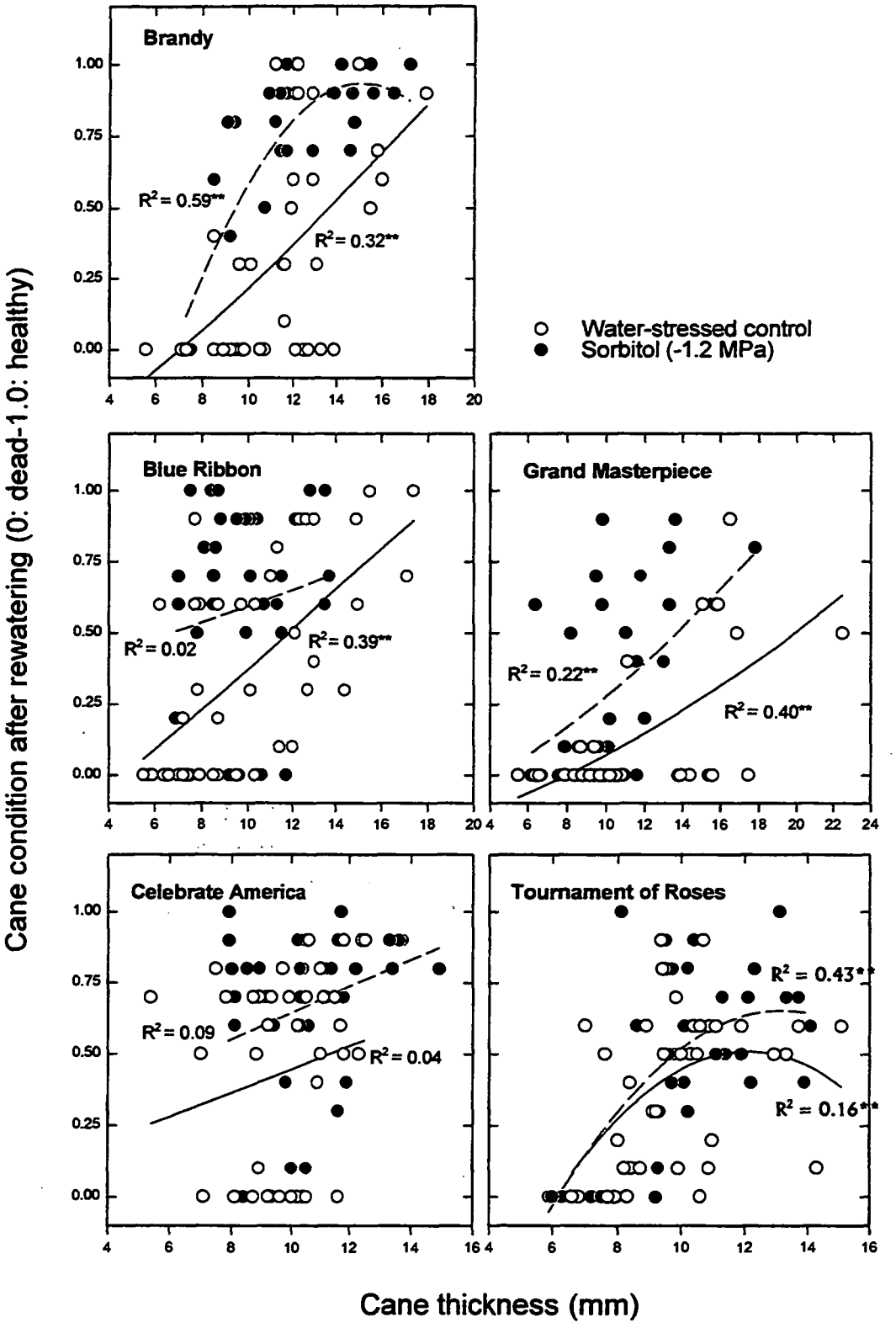
Brandy
R² = 0.59**
R² = 0.32**
Water-stressed control
Sorbitol (-1.2 MPa)
Blue Ribbon
R² = 0.02
R² = 0.39**
Grand Masterpiece
R² = 0.22**
R² = 0.40**
Celebrate America
R² = 0.09
R² = 0.04
Tournament of Roses
R² = 0.43**
R² = 0.16**
Cane condition after rewatering (0: dead-1.0: healthy)
Cane thickness (mm)

Figure F5

Figure F6. Shoot development 14 days after treatment (A), shoot wilting 48 days after water stress (B), shoot recovery 25 days after rewatering (C), and flowering 40 days after rewatering (D) in a cultivar 'Celebrate America' of bagged-roses treated with either water or -1.2 MPa of sorbitol solution as a drench to the medium and then exposed to desiccation stress.

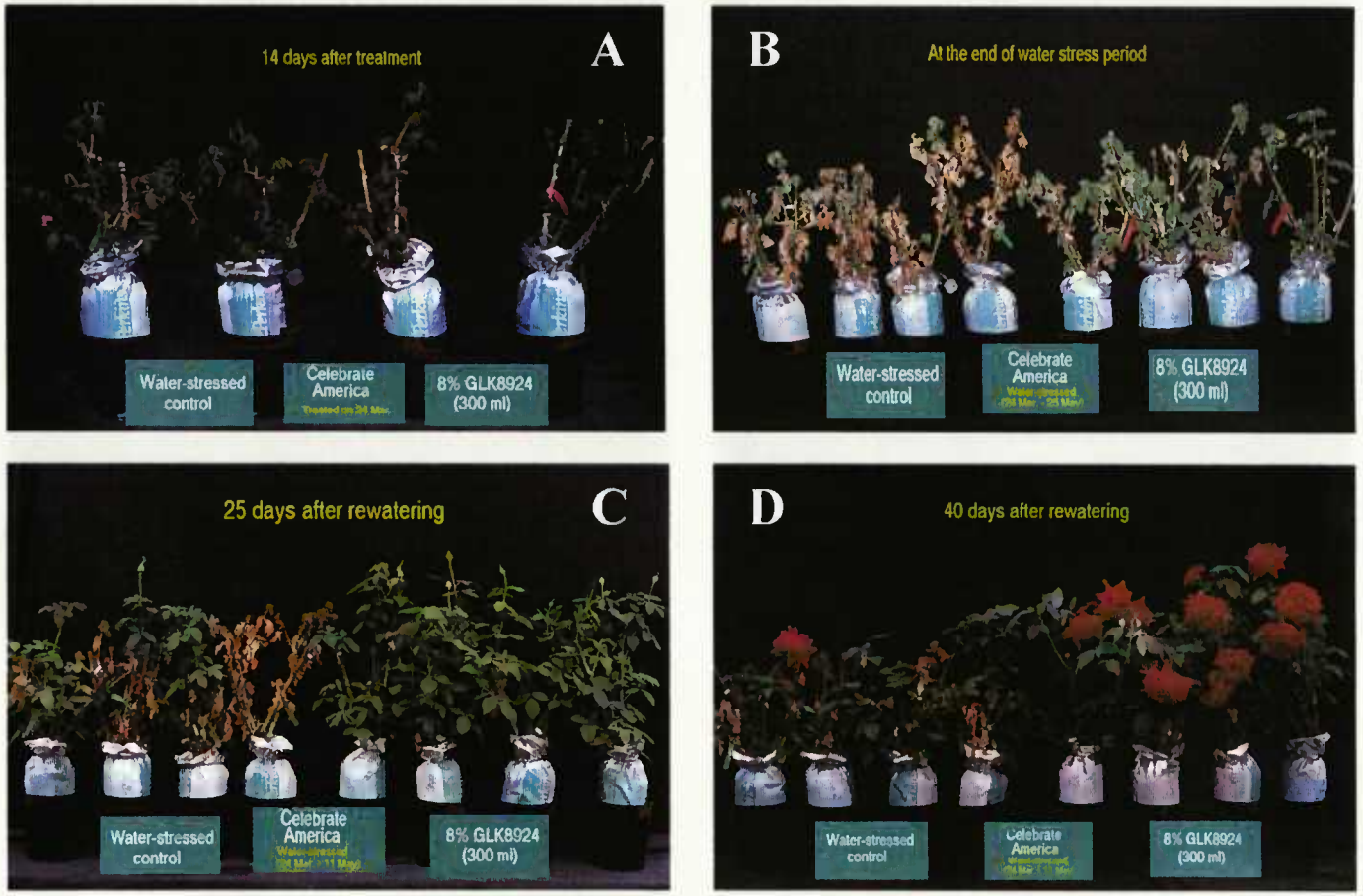
14 days after treatment
A
Water-stressed control
Celebrate America
8% GLK8924 (300 ml)
B
At the end of water stress period
Water-stressed control
Celebrate America
8% GLK8924 (300 ml)
25 days after rewatering
C
Water-stressed control
Celebrate America
8% GLK8924 (300 ml)
D
40 days after rewatering
Water-stressed control
Celebrate America
8% GLK8924 (300 ml)

Figure F6

APPENDIX G

REDUCING WATER LOSS, STORAGE, ESTABLISHMENT, AND COLD HARDINESS OF CONTAINERIZED DOUGLAS-FIR AND WESTERN HEMLOCK SEEDLINGS

G.1 Abstract

The objective of this study was to determine the effect of sorbitol-induced osmotic stress (SIOS) on reducing water loss and cold hardiness of Douglas-fir and hemlock plug seedlings. These studies were performed by Oregon State University and Weyerhaeuser researchers. Oregon State University researchers studied the effects of SIOS on desiccation resistance of Douglas-fir and hemlock plug seedlings under controlled greenhouse experiments when applied before and after cold storage, and cold hardiness of Douglas-fir seedlings. Weyerhaeuser researchers studied the effects of SIOS on desiccation resistance of Douglas-fir and hemlock plug (and mini-plugs of hemlock) when applied before and after cold storage under greenhouse and field conditions.

Application of sorbitol (-0.75 and -1.5 MPa) as a drench to the root system either before or after cold storage was effective in reducing water loss in both Douglas-fir and hemlock plug seedlings under greenhouse tests at Oregon State University and Weyerhaeuser Co. Both concentrations of sorbitol had similar effects on reducing water loss, with the higher level being slightly more effective. Recovery of plants and root growth potential of plants from desiccation stress was significantly better in the SIOS-treated plants than the water-stressed controls. Douglas-fir seedlings recovered at higher percentages than hemlock seedlings.

In field tests, SIOS treatments before or after cold storage to Douglas-fir and hemlock plugs and hemlock mini-plugs significantly reduced the height of the seedlings and delayed the time of bud flush of the seedlings (no data on mini-plugs). At the one month assessment period, the percentage of low vigor and/or dead plants were significantly higher in the -1.5 MPa treatment when applied to plants prior to cold storage in both species and at the -1.5 MPa treatment applied after cold storage to the Douglas-fir seedlings only. The percentage of packability in Douglas-fir seedlings was reduced by

SIOS treatment, particularly when application was made before cold storage. In hemlock, the SIOS treatment reduced the percent of packability when application was made at the -1.5 MPa level before cold storage. In all treatments, no beneficial effect was found in improvements of the growth and survival of all plants tested when treated with sorbitol.

The cold hardiness of Douglas-fir plug seedlings obtained from Rochester, WA greenhouses and field grown seedlings from Aurora Nursery was not affected by SIOS treatments according to controlled laboratory freezing studies.

G.2 Introduction

The researchers at Oregon State University (Fuchigami and Guak) were given the opportunity to test GLK8924 whose main ingredient is sorbitol, produced by the Great Lakes Chemical Corp. (West Lafayette, IN) for use of reducing water loss in plants. Preliminary tests conducted on numerous plant species; including temperate woody plants (e.g., deciduous and coniferous evergreen plants), tropical, vegetables, and herbaceous annuals and perennials; showed conclusively that sorbitol (GLK8924)-induced osmotic stress (SIOS) by drench reduced water loss in containerized and field grown plants. The mechanisms of action of SIOS in reducing water loss is not well understood. However, preliminary experiments showed that SIOS induces ABA production and stomatal closure in containerized apple trees within two hr after treatment, and that SIOS-induced stomatal closure was closely associated with the concurrent reduction in leaf turgor. ABA production and stomatal closure is concentration dependent and species specific. Generally herbaceous plants require less sorbitol concentration than woody plants to induce stomatal closure. The effect of SIOS on stomatal closure can be duplicated by application of either ABA or ABA analogs, suggesting further that ABA may be the cause of SIOS-induced stomatal closure.

Preliminary tests on Douglas-fir, Western hemlock, spruce, and cedar indicated that SIOS reduced water loss by inducing stomatal closure. Treated plants survived dehydration stress several days and weeks longer than the untreated controls. When used at the optimum concentration range no phytotoxicity was observed. At high

concentrations, SIOS can cause growth retardation without causing symptoms of phytotoxicity. Plants rehydrated and recovered after SIOS and water stress treatment perform satisfactorily with no visual signs of damage caused by the chemical.

In apples, containerized plants treated with SIOS, following a freeze in December that caused damage to the cortical and cambial tissues of stems located near the soil surface, recovered without any signs of tissue damage while the untreated controls were all damaged. Plants were moved into a warm greenhouse at several time periods after the freeze event and treated by drenching the soil with either water or various concentrations of sorbitol. All plants treated with SIOS survived while the untreated plants all died or showed severe damage to the tissues located near the soil surface. The reason why treated plants survived is not known. These tests are difficult to repeat, however, the consistency of the data suggests that SIOS did enhance the recovery of these plants.

There is no evidence that SIOS affects the freezing resistance of plants. Research to date with this compound has focused primarily on regulation of stomatal closure, water reduction and retardation of plant growth. Because of the close association between water stress and cold hardiness there is a tendency to believe that this compound may have an effect on the freezing resistance of plants.

The objectives of this project were as follows:

Task A. Core Feasibility Study:

- Determine whether SIOS increases desiccation resistance of greenhouse and container grown Douglas-fir and Western hemlock seedlings treated before and after cold storage.
- Evaluate the direct and indirect impacts of SIOS on the freezing resistance of Douglas-fir seedlings.

Task B. Cooperative Studies at Weyerhaeuser Company:

- Assist researchers at Weyerhaeuser Company to evaluate the impact of

SIOS on desiccation resistance and transplant establishment of Douglas-fir and hemlock seedlings produced in the greenhouse, cold stored and planted into the nursery bed outdoors.

- Assist researchers at Weyerhaeuser Company to evaluate the impact of SIOS on freezing resistance of Douglas-fir seedlings at the Aurora Nursery.

G.3 Materials and Methods

Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco. DF/104 R-T03-4] and Western hemlock [*Thuga heterophylla* (Raf.) Sarg. MP R-T11-4 (Blue M) and Styroblock R-TS-4 (Black K)] seedlings (plug +1), grown in plug containers at greenhouses at Rochester, WA., were used for the majority of the core feasibility studies conducted at Oregon State University and the cooperative studies at Weyerhaeuser Company. The seedlings were obtained from the Rochester nursery on December 14, 1994. The seedlings were grown as plugs in the greenhouses at Rochester. The seedlings were extracted from the containers with the media intact before storage and treatment as discussed below. The seedlings were divided into two batches for use at either Oregon State University or Weyerhaeuser Company.

G.3.1 Studies at Oregon State University

Storage and desiccation resistance tests: On December 21 1994, the Douglas-fir and Western hemlock seedlings were further divided into two groups at Oregon State University:

1) Group A (pre-storage treatment) seedlings were treated with either water, -0.75, or -1.5 MPa of sorbitol by soaking the root system and medium of ten plants each in plastic containers containing 1.5-liters of the above solutions for 18 hr in a greenhouse set at 25°/18°C (day/night) temperature and natural light. After soaking the media the excess solution was permitted to drain. After draining the treatments were further divided into 3 sub-groups:

a. Sub-group 1 was placed immediately into 1.25" x 5.0" plugs in the greenhouse.

b. Sub-group 2 was wrapped with saran and placed in a cardboard box at -2°C for 50 days, removed from storage and transplanted into 1.25" x 5.0" plugs and placed in the greenhouse as subgroup 1.

c. Sub-group 3 was wrapped with saran, placed in a cardboard box at -2°C for 83 days, removed from storage and transplanted in 1.25" x 5" plugs and placed in the greenhouse as in sub-group 1 and 2.

2) Group B (post-storage treatment) seedlings were treated with water only as above, wrapped with saran, placed in a cardboard box at -2°C for 50 and 83 days. Immediately after the storage period the seedlings were treated with either water, -0.75, or -1.5 MPa of sorbitol as above. After soaking the seedlings were placed into 1.25" x 5.0" plugs as above and placed in the greenhouse conditions described above.

The following data were obtained from the above treatments for both species: 1) stem diameter and plant height; 2) relative weight change after 0, 2, 4, 6 and 8 days after treatment and placement in the greenhouse; and 3) percentage of plants recovered after rewatering. The data were analyzed by analysis of variance and least significant difference (LSD) tests.

Cold hardiness tests: Three cold hardiness tests were conducted by Oregon State University researchers:

1) In the first test, the Douglas-fir plants obtained from Rochester, WA were transplanted into 1.25" x 5.0"plugs, treated with either water, -0.75, or -1.5 MPa of sorbitol solution in December 24, 1994. After treatment the plants were placed under natural conditions at Corvallis, OR for 1, 2, or 4 weeks prior to the freezing test. The plants were subjected to an artificial freeze test in a programmable Forma Scientific freezer (Model: 8770 S/N 83875-45). To prevent freezing of the root system, the tops of the plants were passed through a small opening in a 2.54 cm thick Styrofoam lid so that the root system in the growing media and plugged container was outside of the freezer and the tops inside of the freezing chamber. Therefore, the root system was never exposed to temperatures below zero degree Celsius.

The test temperatures were +4°, -2°, -5°, -8°, -11°, -14°, -17° and -20°C. The freezing rate was 3°C per hr and the tissues were exposed to one hr at each test temperature before removal from the freezer. After removal, the plants were incubated at 4°C overnight. Thereafter the plants were placed in a greenhouse at 25°/18°C (day/night) and natural light. The plants were evaluated after one month. No differences were found between treatment. All plants (needles) died at temperatures lower than -8°C at all test dates, suggesting that the plants were not acclimated at the time of the study. The SIOS had no effect on hardiness.

2) In the second test, 1D0 Douglas-fir seedlings growing under natural conditions in nursery beds at the Weyerhaeuser Aurora Nursery were treated with either water, -0.75, or -1.5 MPa of sorbitol. The nursery beds containing the plants were divided into 3 feet sections per treatment with a buffer of one foot between treatments. Each treatment was replicated 4 times at random within one nursery bed. The treatments were drenched into the beds with a watering can on February 10 1995. Twenty-liters of solution were applied per treatment. This study was done in collaboration with Rod Miller, who was employed by Weyerhaeuser at the time of this study. There were no differences observed in the performance (based on appearance) of the treated and control plants.

3) In the third test, 1D0 Douglas-fir seedlings were bare-rooted from the Aurora Weyerhaeuser Nursery on September 27 1995 and transported to Corvallis, OR. The plants were transplanted into fiber 1.5-gallon containers with a mixture of 1:1:1:1 (by volume) soil, pumice, peat, and perlite. After potting the plants were placed under natural light and temperature conditions at the OSU West greenhouse range (outdoors) at Corvallis, OR. On February 3, 1996, the following treatments were applied by drenching 500 mls of solution per container per treatment: water controls, -0.6, and -1.2 MPa of sorbitol. After application of the compound the plants were placed in a controlled greenhouse at 25°/18°C (day/night) greenhouse under natural light. The plants were replicated at random with 10 plants per treatment.

At 5 and 10 days after treatment controlled freezing tests were performed in the Forma Scientific programmable freezer. Previous tests on Douglas-fir by Oregon State researchers and Roger Timmis (Sue Fisker) suggest that the best tissue for evaluating

hardiness of Douglas-fir was the previous season's needles. Therefore, the needles of Douglas-fir were removed from the previous seasons by hand and 15 needles per replicate were placed uniformly onto a layer of aluminum foil, lined with cheesecloth and sprayed with water to induce nucleation during freezing. The needles and cheesecloth were then enclosed within the aluminum sheet by folding the aluminum sheet over the cheesecloth and hand-sealing the edges of the aluminum sheet. Each replicate was wrapped in a separate packet and randomized on the aluminum block in the freezer. The samples were placed in the freezer at -2°C overnight prior to the freezing run. The freezing rate was 3°C per hr and the test temperatures were -5°, -11°, -17°, -23°, -26°, and -32°C after 5 days and -5°, -11°, -17°, -20°, -26°, and -29°C after 10 days of incubation. The tissues remained at the each test temperature for 30 min, after which they were removed from the freezer and placed at 4°C overnight. The samples were then placed at room temperature for 7 days and evaluated for injury by visual observation. The evaluation of needle injury was based on the following numerical system: 0=no damage (0% index of injury), 1=slight discoloration, 2=severe discoloration, and 3=all tissues dead (100% index of injury).

The percent index of injury was evaluated by the Jandel Scientific Peakfit (Jandel Scientific, San Rafael, CA) software program to determine the Lt40 (temperature resulting in 40% injury). Statistical differences will be determined by standard deviation.

G.3.2 Studies at Weyerhaeuser Company

G.3.2.1 Desiccation tests in the greenhouse

Hemlock: Western hemlock (Ref. #RTS2-4) seedlings produced in styroblocks (Styro 4A 3.6 cu in per cell) at Rochester, WA were treated with either water, -0.75, or -1.5 MPa of sorbitol on January 13 1995. Each plant was treated with a total of 30 mls of the above solutions by dispensing 15 mls each two times per cell with an automatic pipetter. A total of 1000 seedlings were treated per treatment. Fifty seedlings from each treatment were divided into equal groups (25 plants each) and the root system was placed individually into either poly tubes or small polyethylene bags. The other plants (950

seedlings per treatment) were placed into cold storage units at Olympia, WA for 112 days.

The plants in the poly tubes and poly bags were placed in a heated greenhouse (≈78°F) at Centralia, WA. The plants were weighed individually at the start of the experiment and daily thereafter. After 16 days of exposure in the above containers the plants were transplanted (4 seedlings each) into 2-gallon poly containers with a peat/perlite mix. Each container, with 4 seedlings, was treated as a replicate. The plants were watered thereafter and grown in the greenhouse. The recovery and root growth potential (RGP) of the plants were determined after 4 weeks.

Hemlock mini-plugs: Western hemlock seedlings (Ref. #R-T11-4) produced in mini-plugs (Miniplugs, 0.56 cu.in. per cell) at Rochester, WA were treated with the same concentrations above by drenching the entire tray into the solutions. The solution (1,200 mls) was placed into plastic trays and the miniplugs were agitated in the solution for 10 minutes, removed and allowed to drain.

Douglas-fir: Douglas-fir seedlings (Ref. # R-T03-4) produced in white multipot 5 trays (3.0 cu. in. per cell) at Rochester, WA were used for the following studies. The same treatments described above for Western hemlock were used for these studies. The plants were treated on January 17 1996 and transplanted into the 2 gallon containers after 12 days of desiccation in the greenhouse. Each container contained 5 seedlings and was treated as a single replicate as above. Recovery and RGP were determined 4 weeks after transplanting and placement into the greenhouse environment.

G.3.2.2 Storage, greenhouse, field tests for desiccation resistance

Storage and greenhouse test: Douglas-fir (DF/104 R-T034) and Western hemlock (MP R-T11-4 and styroblock R-TS2-4) produced as plugs +1 seedlings treated before storage (112 days for Hemlock and 116 days for Douglas-fir) with the either water, -0.75, or -1.5 MPa of sorbitol solutions (see above for application procedure). The duration of the desiccation study in the greenhouses for Western hemlock and Douglas-fir seedlings was 16 and 12 days, respectively. After the desiccation test, the plants were transplanted into 2-gallon containers as above and evaluated after 4 weeks in the greenhouse. The

following data was collected from this study: change in weight during desiccation treatment in greenhouse and root growth potential and viability of plants.

Storage and field (Mima nursery) test: Douglas-fir plugs, Western hemlock plugs and Western hemlock mini-plug seedlings [900-950 seedlings per treatment (0, -0.75, and -1.5 MPa of sorbitol solutions) from the pre-storage treatments and 3,000 seedlings without pretreatment for each group] were removed from cold storage and treated between 27-28 April 1995 with the post-storage treatments (0, -0.75, or -1.5 MPa of sorbitol solutions). The seedlings were shipped to Mima nursery and planted on 1 May 1995. In addition to the pre- and post-storage treatments the Weyerhaeuser operational control (no water soak-plants planted directly from cold storage) was included as a separate treatment. The treatments were divided into 4 replications each containing between 225-238 plants. The Douglas-fir and the styroblock grown hemlock seedlings were planted in 7 rows, and the mini-plug hemlock seedlings were grown in 8 rows. The experimental design was a randomized complete-block design.

At the nursery the following observations were made on the dates indicated:

- May 8, 1995: Plant vigor and foliage retention.
- May 31: Plant vigor, bud flush and foliage retention.
- June 1: Percent bud flush of Douglas-fir
- June 19: One month assessment (% dead and low vigor) of Douglas-fir.
- July 31: Mid-season assessment (height and caliper) of Douglas-fir.
- May 25: Percent bud flush of hemlock plugs.
- June 15: One month assessment (% dead and low vigor) of hemlock plugs.
- August 2: Mid season assessment (height) of hemlock plugs.
- June 11: One month assessment (% dead and low vigor) of hemlock mini-plugs.
- August 2: Mid season assessment (height) of hemlock mini-plugs.

- December 15: Packability assessment for all plants.

The statistical analyses included analysis of variance and least significant differences (LSD's).

G.4 Results and Discussion

G.4.1 Greenhouse/storage tests for desiccation resistance

G.4.1.1 OSU studies

Douglas-fir and hemlock seedlings responded similarly to all treatments (Figs. G1 and G2; Table G1). Seedlings treated with SIOS before and after cold storage lost significantly less water than did the untreated controls. No differences were found between the time of SIOS application. The -1.5 MPa sorbitol treatment was slightly more effective than the -0.75 MPa treatment in reducing water loss, however, plants treated with the -1.5 MPa sorbitol solution showed some symptoms of wilting immediately after treatment. The plants treated with the -1.5 MPa solution recovered from the initial wilt symptoms and performed as well as the -0.75 MPa treatment.

Rewatering of Douglas-fir, 7-8 days after the desiccation treatment, to determine the recovery of plants exposed to desiccation stress, indicated significant differences between the desiccated controls and SIOS treatments. None of the water-stressed controls survived (Table G1 and Fig. G1B). In contrast, between 70-100% of the SIOS-treated plants recovered whether treated before or after cold storage. There were no significant differences observed between plants treated either before or after cold storage, and concentrations of SIOS. The appearance of the SIOS-treated plants were similar to the well-watered control plants.

Although there were little or no significant differences in plant height and stem caliper between the treatments, in general, the plants treated with SIOS were slightly smaller than the well-watered control plants (Table G1). The only significant difference in plant height between the well-watered control and SIOS-treated plants was at the -1.5 MPa sorbitol concentration applied after 50 days of cold storage.

Table G1. Recovery and post-growth of Douglas-fir assessed approximately 6-9 months after desiccation stress (8, 9, and 8 days for 0, 50, and 83 days cold storage, respectively). Seedlings were treated before (Pre) or after (Post) storage with either water, -0.75 MPa, or -1.5 MPa of sorbitol solution. Seedlings treated or untreated were stored at -2°C for 0, 50, or 83 days.

	Survival (%)			Height (cm)			Stem diameter (mm)		
	Cold storage (days)			Cold storage (days)			Cold storage (days)		
Treatment	0	50	83	0	50	83	0	50	83
Well-watered control	100.0 a[z]	100.0 a	100.0 a	24.2 a	25.3 a	25.4 a	5.7 a	5.2 a	5.2 a
Water-stressed control (Pre)	0.0 b	0.0 c	0.0 b	-	-	-	-	-	-
Water-stressed control (Post)	-	0.0 c	0.0 b	-	-	-	-	-	-
Sorbitol (-0.75 MPa) (Pre)	100.0 a	90.0 ab	90.0 a	24.0 a	23.4 a	24.3 ab	5.4 a	5.2 a	5.1 a
Sorbitol (-0.75 MPa) (Post)	-	90.0 ab	100.0 a	-	23.6 a	22.1 ab	-	5.1 a	5.1 a
Sorbitol (-1.5 MPa) (Pre)	100.0 a	80.0 ab	90.0 a	18.1 b	24.0 a	21.0 ab	5.2 a	5.0 a	4.9 a
Sorbitol (-1.5 MPa) (Post)	-	70.0 c	100.0 a	-	23.2 a	20.0 b	-	5.1 a	5.2 a

[z] Same letters within a column mean no significant difference in means, separated by Duncan's multiple range test at $P = 0.05$.

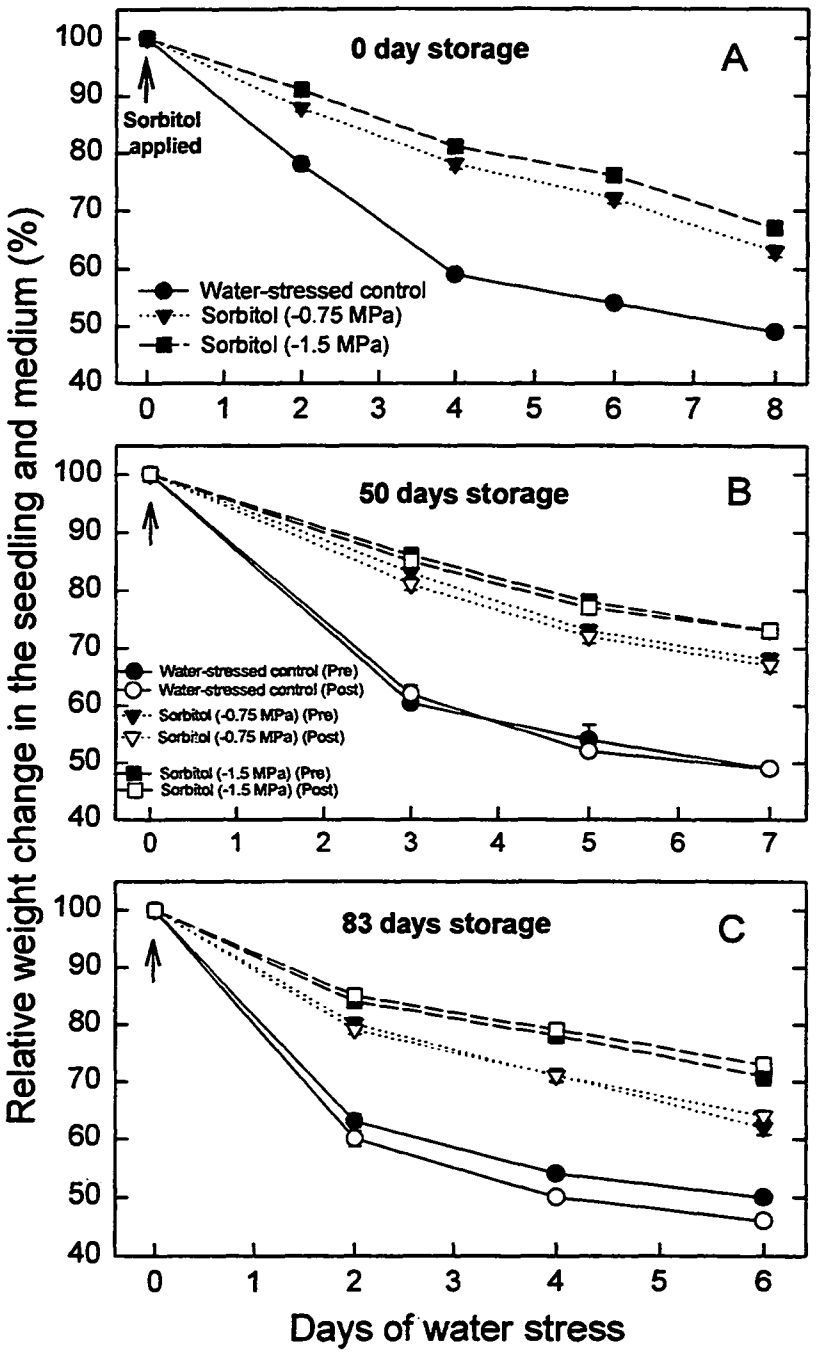

Figure G1A. Relative weight change during desiccation stress period in Douglas-fir seedlings (+media) treated with either water, -0.75, or -1.5 MPa of sorbitol solution before or after cold storage for 0, 50, and 83 days.

Figure G1B. Douglas-fir seedlings recovered and grown after rewatering. Seedlings were treated with either water, 5%, or 10% sorbitol as a soil drench before (pre) or after (post) cold storage for 50 (A) and 83 (B) days, and then exposed to desiccation stress.

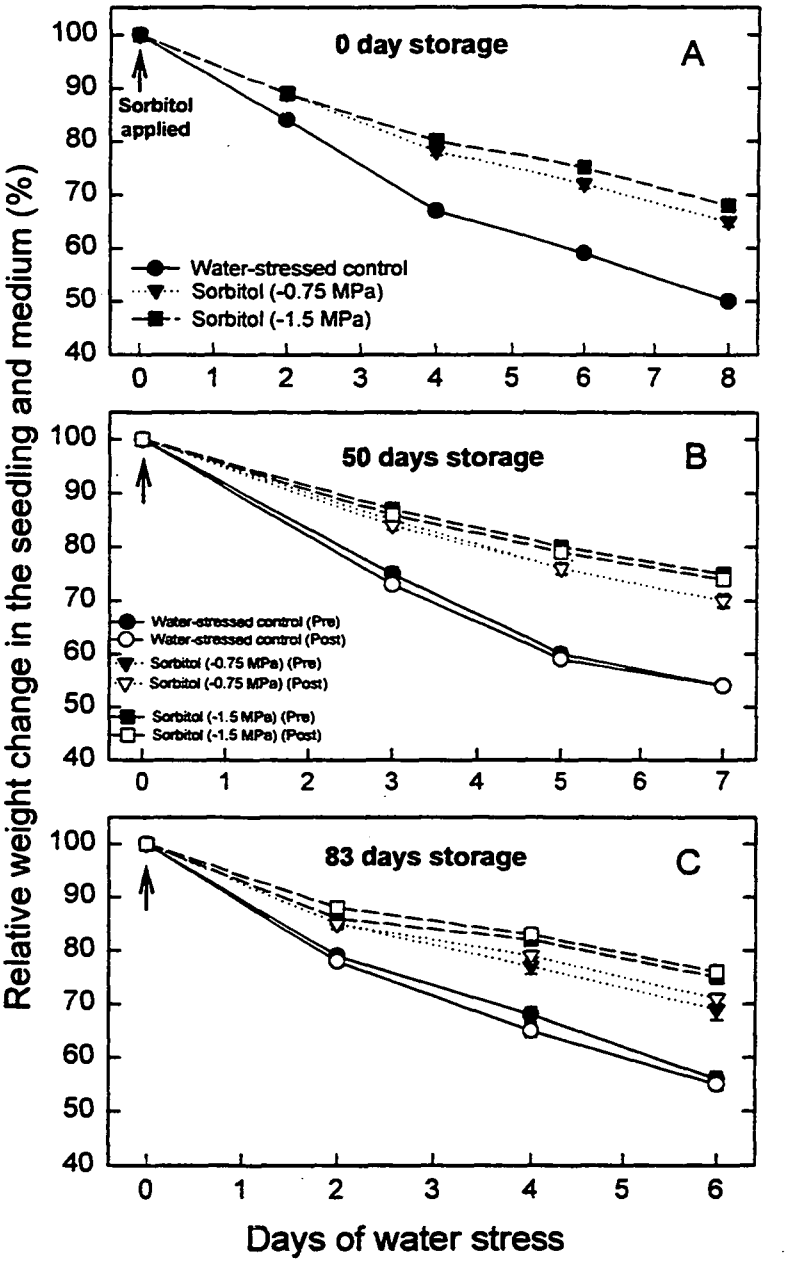

Figure G2. Relative weight change during desiccation stress period in Western hemlock seedlings (+media) treated with either water, -0.75, or -1.5 MPa of sorbitol solution as a drench before or after cold storage for 0, 50, and 83 days.

G.4.1.2 Weyerhaeuser studies

The results of the desiccation tests performed at the greenhouses at Centralia, WA on Douglas-fir and hemlock seedlings were similar to the results obtained at OSU (Figs. G3, G4, and G5). In contrast to the results obtained at OSU, significant differences were found between -0.75 and -1.5 MPa SIOS treatments, with the latter being more effective in reducing water loss (Figs. G4 and G5). Both concentrations were effective in significantly reducing water loss in both species. In the desiccation test, plants grown in open tubes, with the original media only, did not do as well as those grown in Rochester promo (poly) bags (Figs. G3 and G4).

The recovery (viability, bud flush, buds alive, buds dead, cambium condition, needle condition) of the plants 4 weeks after the desiccation stress treatment were similar to the results obtained in the OSU study (Tables G2 and G3). Both Douglas-fir and hemlock seedlings treated with SIOS recovered at significantly higher rates than the controls. Douglas-fir seedlings recovered better than hemlock seedlings. The effect of SIOS treatment before cold storage was not concentration dependent in both species (Table G2), whereas treatment made after cold storage in Douglas-fir only improved with the higher concentration (Table G3).

The production of new roots was also significantly greater in the SIOS treatments of both plant species (Tables G2 and G3). The effect of SIOS was not concentration dependent, however, there was a tendency for the higher concentration to reduce the number of new roots when applied before storage and increase the numbers of new roots after cold storage.

Table G2. Root growth potential on Douglas-fir and Western hemlock plug seedlings assessed 4 weeks after desiccation stress period (8 or 10 days for Douglas-fir and 11 days for hemlock). Seedlings were treated with either water, -0.75 MPa, or -1.50 MPa of sorbitol solution just before desiccation stress.

Species	Desiccation stress Period	Treatment	New root (≥cm)	Buds flushed	Buds alive	Cambium condition	Cambium damage location	Needle damage	Viability
Douglas-Fir	8 days	Control	-	0.00 b[z]	-	0.00 b	6.0 a	-	-
		Sorbitol (-0.75 MPa)	-	0.00 b	-	0.00 b	6.0 a	-	-
		Sorbitol (-1.50 MPa)	-	0.20 a	-	0.20 a	4.8 b	-	-
	10 days	Control	0.70 b	0.10 b	0.55 b	0.05 b	5.4 a	4.50 a	0.05 b
		Sorbitol (-0.75 MPa)	11.10 a	0.45 a	4.05 a	0.40 a	3.6 b	3.10 b	0.40 a
		Sorbitol (-1.50 MPa)	8.89 a	0.56 a	4.89 a	0.50 a	3.0 b	2.39 b	0.50 a
Western Hemlock	11 days	Control	0.00 b	0.00 b	0.00 b	0.00 b	6.0 a	5.00 a	0.00 b
		Sorbitol (-0.75 MPa)	7.30 a	0.24 a	2.43 a	0.24 a	4.5 b	3.92 b	0.24 a
		Sorbitol (-1.50 MPa)	4.46 a	0.22 a	2.27 a	0.22 a	4.5 b	4.70 a	0.22 a

[z] Same letters within a column for each species mean no significant difference in means, separated by Duncan's multiple range test at $P = 0.05$.

Table G3. Root growth potential on stored Douglas-fir and Western hemlock plug seedlings assessed 4 weeks after desiccation stress (12 days for Douglas-fir and 16 days for hemlock). Seedlings were treated with either water, -0.75 MPa, or -1.5 MPa of sorbitol solution approximately 15 weeks after cold storage.

Species	Treatment	New root (≤cm)	New root (≥cm)	Bud Flush	Buds alive	Buds dead	Cambium condition	Needle condition	Viability
Douglas-Fir	Control	0.00	0.00 b	0.00 c	0.00 b	4.92 a	0.00 b	5.00 a	0.00 b
	Sorbitol (-0.75 MPa)	0.00	15.92 a	0.68 b	4.92 a	1.60 b	0.68 a	2.12 b	0.68 a
	Sorbitol (-1.50 MPa)	0.00	17.20 b	0.92 a	4.84 a	0.56 b	0.92 a	1.24 b	0.92 a
Western hemlock	Control	0.00 a	0.00 b	0.00 b	0.00 b	10.00 a	0.00 b	5.00 a	0.00 b
	Sorbitol (-0.75 MPa)	1.00 a	5.64 a	0.36 a	3.52 a	6.40 b	0.40 a	3.48 b	0.36 a
	Sorbitol (-1.50 MPa)	1.68 a	7.76 a	0.36 a	3.84 a	6.00 b	0.48 a	3.12 b	0.40 a

[z] Same letters within a column for each species mean no significant difference in means, separated by Duncan's multiple range test at $P = 0.05$.

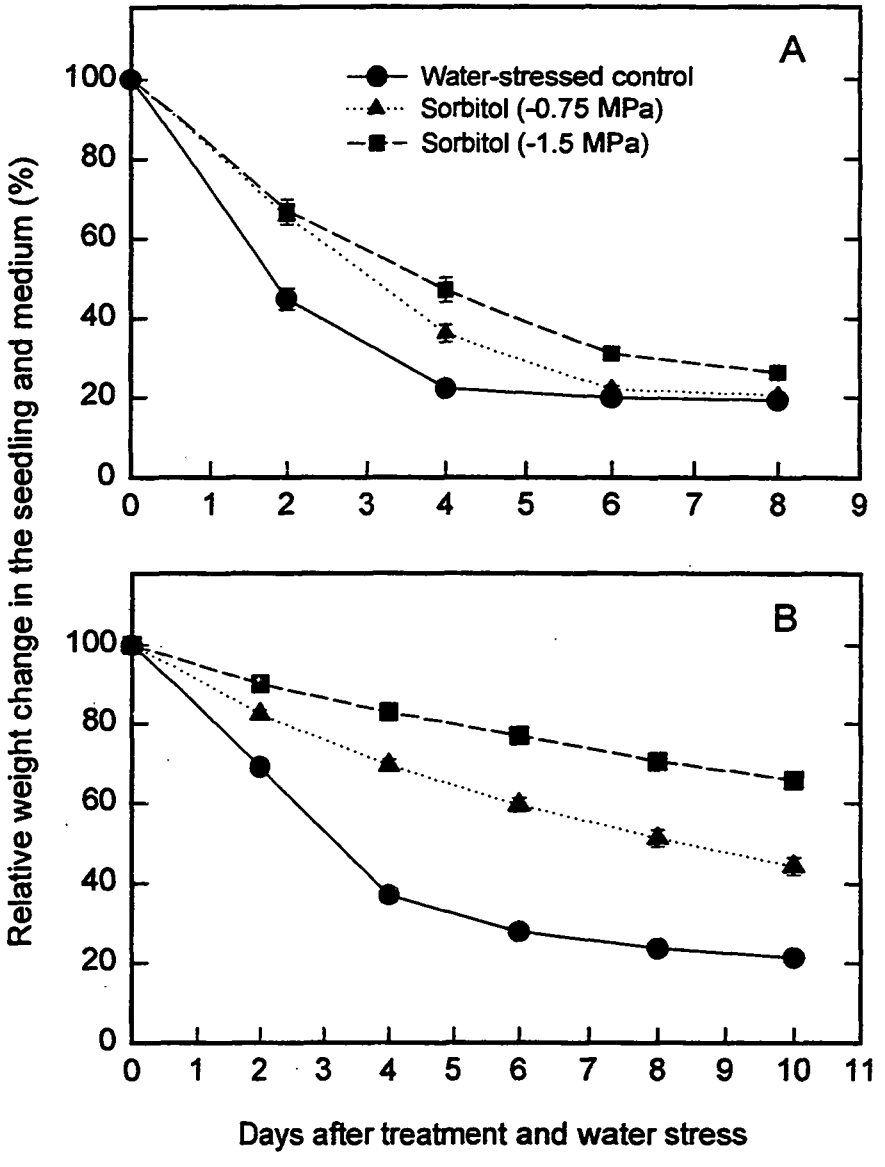

Figure G3. Relative weight change during desiccation stress period in Douglas-fir seedlings (+media) treated with either water, -0.75, or -1.5 MPa of sorbitol solution and placed in plugs (A) and Rochester promo bags (B).

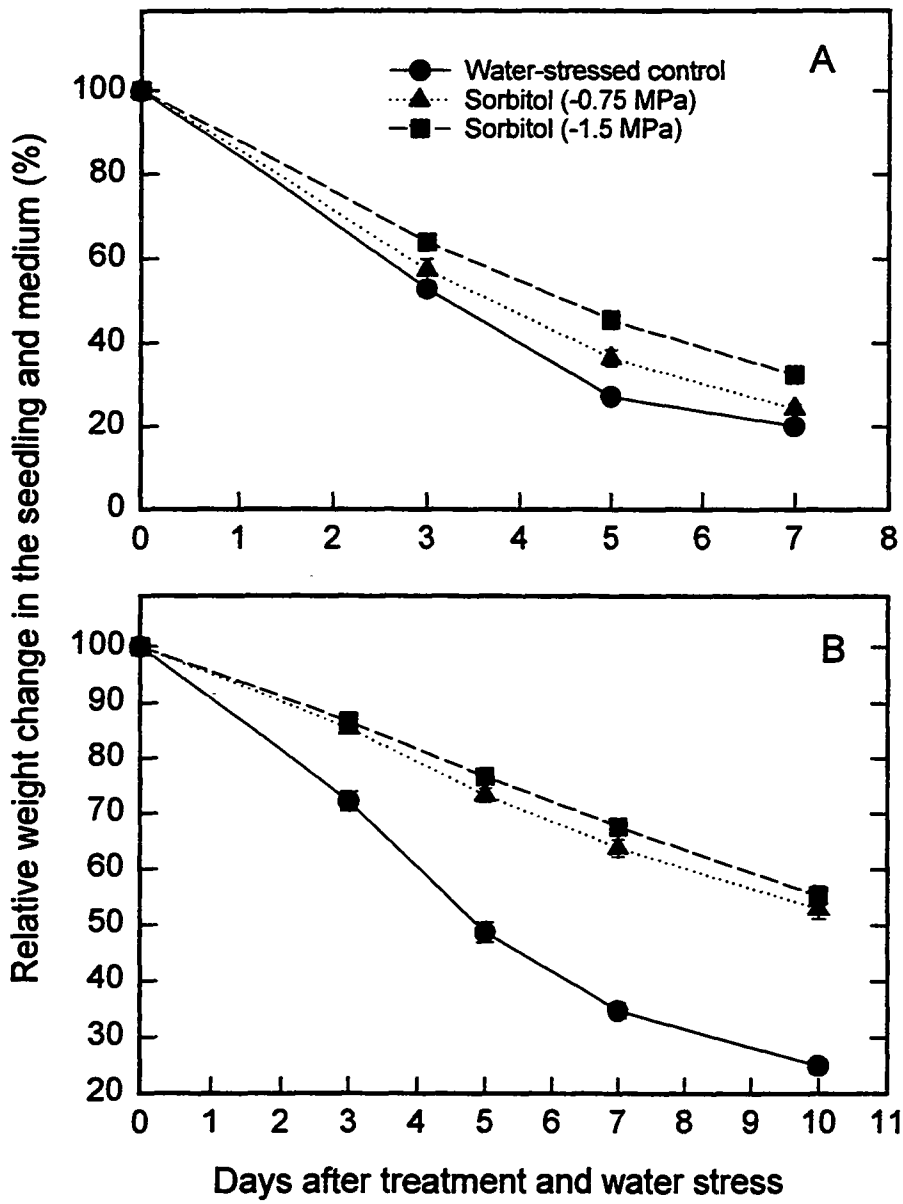

Figure G4. Relative weight change during desiccation stress period in Western hemlock seedlings (+media) treated with either water, -0.75, or -1.5 MPa of sorbitol solution and placed in plugs (A) and Rochester promo bags (B).

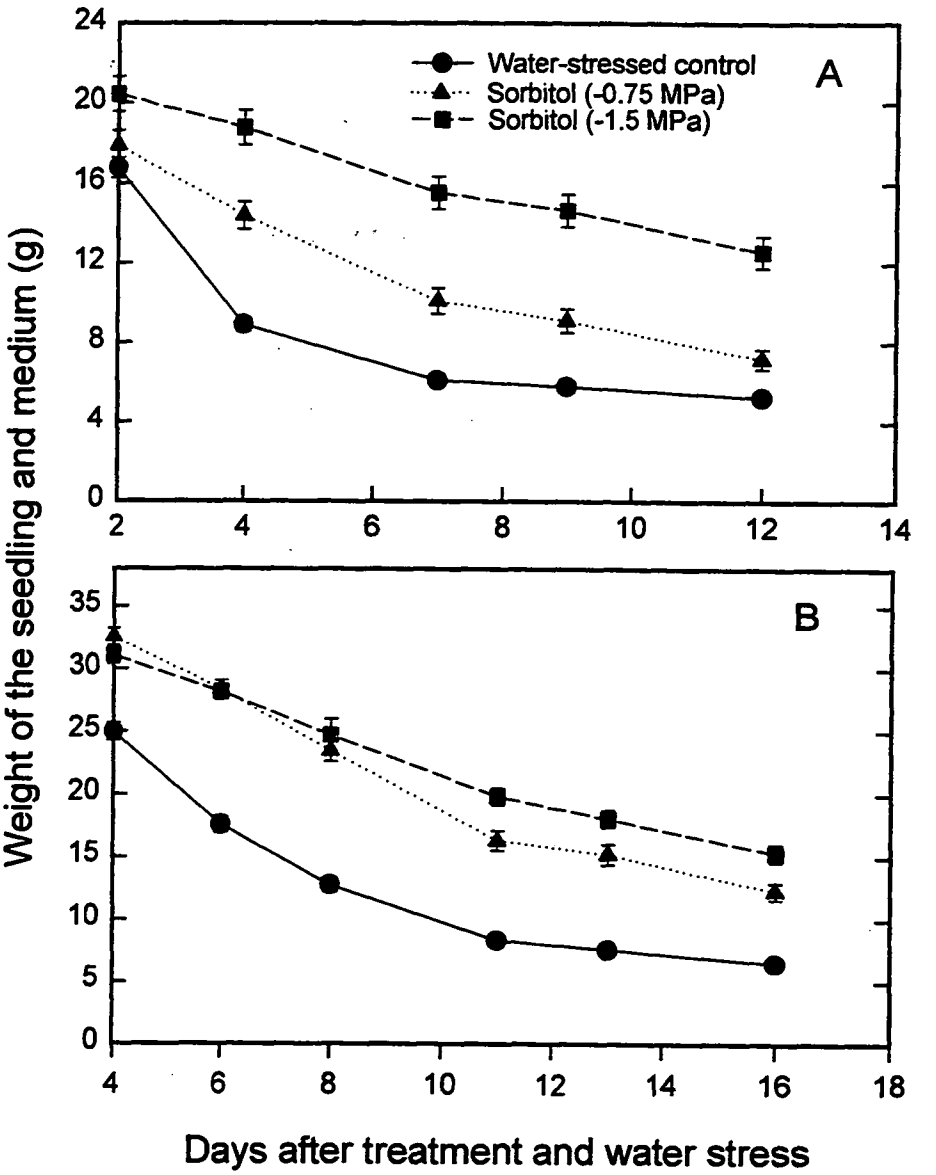

Figure G5. Relative weight change during desiccation stress period in Douglas-fir (A) and Western hemlock (B) seedlings (+media) treated with either water, -0.75, or -1.5 MPa of sorbitol solution after cold storage for 112 days for Western hemlock and 116 days for Douglas-fir) and placed in Rochester promo bags.

G.4.2 Cold storage and field studies performed at Mima Nursery

G.4.2.1 Dieback and vigor

Douglas-fir and hemlock seedlings produced in styroblocks: In general, the extent of dieback or poor vigor, assessed 6-7 weeks after planting, was greater in Western hemlock seedlings than in Douglas-fir seedlings (Fig. G6). In both species, generally plants treated with SIOS appeared to suffered more dieback than the water controls and the operational controls. The amount of dieback was quite low, however, and the only statistically significant difference observed was at the -1.5 MPa SIOS treatment applied before cold storage in both species and, in Douglas-fir at the -1.5 MPa treatment applied after cold storage (Fig. G6). There were no significant differences between the controls

Hemlock mini-plugs: No statistical differences were found between all treatments due to the large variations in dieback and vigor among the mini-plugs (Fig. G7).

G.4.2.2 Bud flush assessment

There were no statistical differences between the three controls (water soaked pre and post-storage and operational no-soak controls) (Fig. G8). In both species, SIOS significantly reduced the percentage of buds flushing during late May, approximately 4 weeks after transplanting at Mima Nursery. The reduction in buds flushing increased with increasing concentration of sorbitol. In general, treatment before of after cold-storage did not appear to affect the per cent of buds flushing, however, significantly fewer buds flushed in hemlock seedlings treated with SIOS before cold storage .

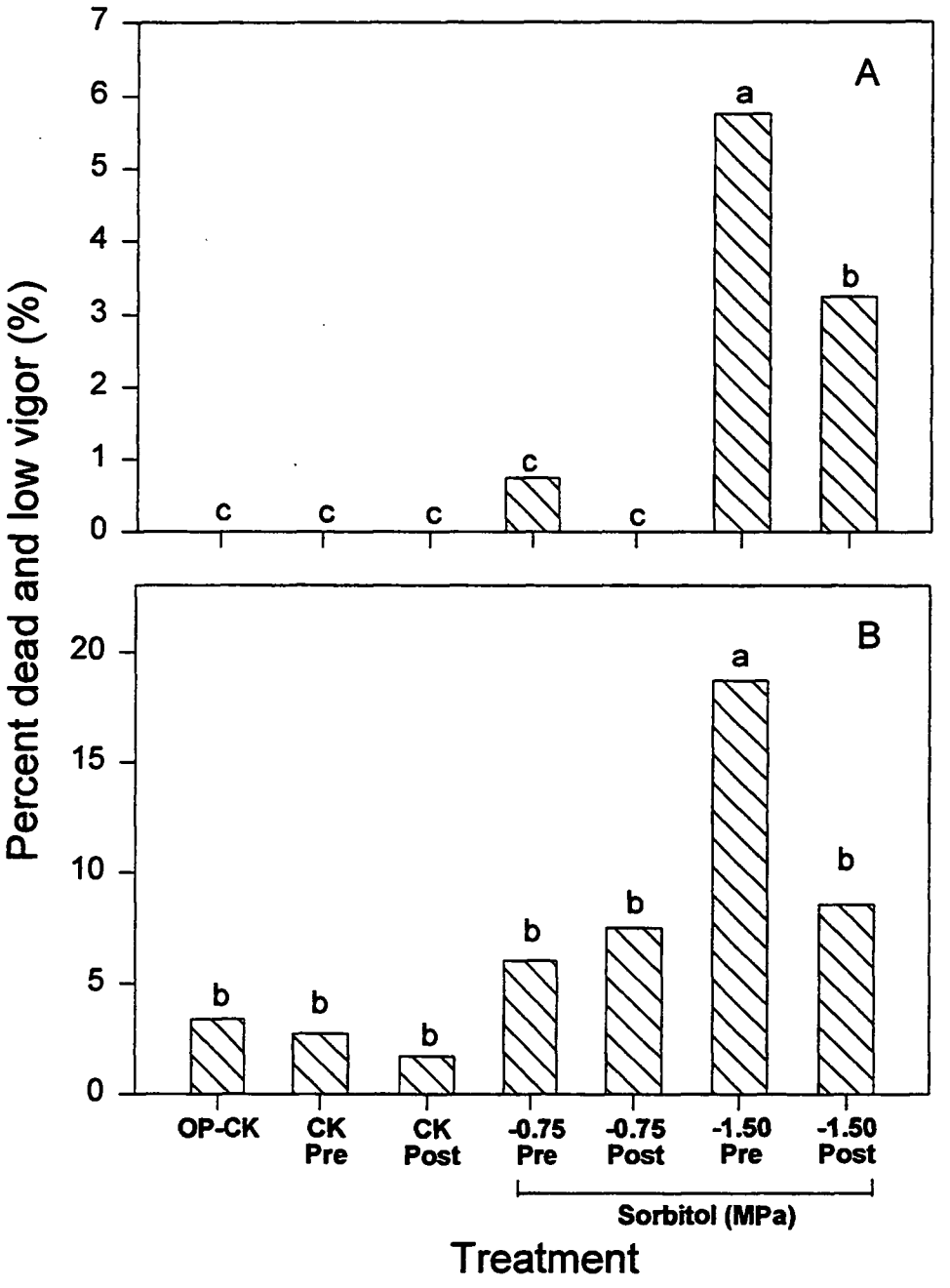

Figure G6. Percent dead or low vigor of Douglas-fir (A) and Western hemlock (B) plug seedlings treated before or after cold storage with either water, -0.75, or -1.5 MPa of sorbitol solution and transplanted on May 1, 1995 at Mima Nursery, approximately after 15 weeks in freezer storage, as assessed during mid June, approximately 6-7 weeks after transplanting. Operational control (OP-CK) is 'no water-soak-plants planted directly from cold storage'.

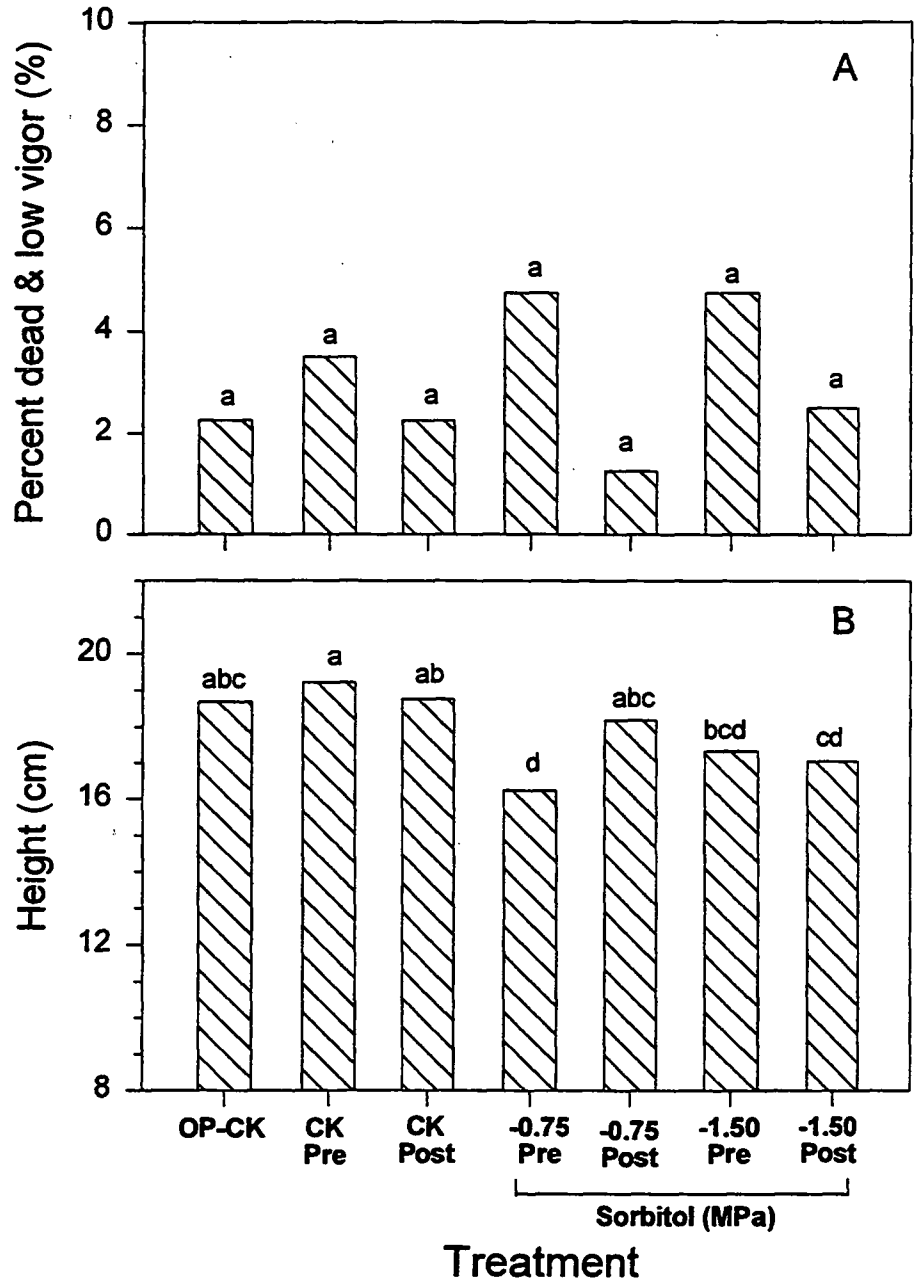

Figure G7. Percent dead or low vigor and height on Western hemlock mini-plug seedlings assessed during mid June, approximately 6 weeks after transplanting at Mima Nursery. Seedlings were treated before or after cold storage with either water, -0.75, or -1.5 MPa of sorbitol solution and transplanted on May 1, 1995 at Mima Nursery, approximately 15 weeks after cold storage.

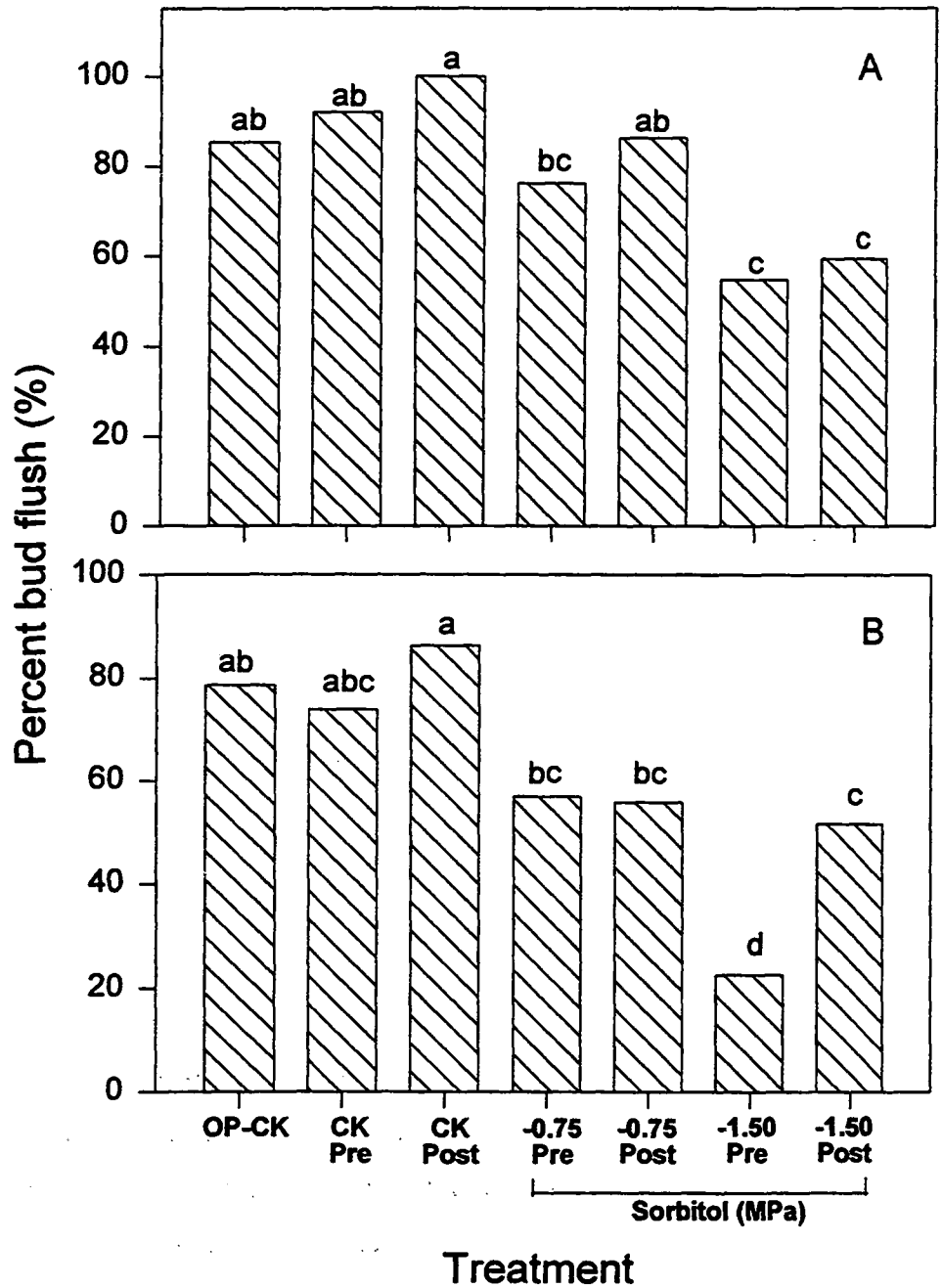

Figure G8. Percent bud flushing assessed during late May, approximately 4 weeks after transplanting at Mima Nursery on Douglas-fir (A) and Western hemlock (B) plug seedlings treated before or after cold storage with either water, -0.75, or -1.5 MPa of sorbitol solution and transplanted at Mima Nursery beds approximately 15 weeks after cold storage.

G.4.2.3 Mid-season assessment of plant height and stem diameter

Douglas-fir and Western hemlock plugs: In general, Douglas-fir plants treated with SIOS were significantly shorter and had a thinner stem diameter than the untreated controls (Figs. G9). Treatment before or after cold storage appeared to have a slight effect on plant height, if any, at the highest concentration only. Generally, plants treated before cold storage were shorter and thinner than the post storage treatments. The control plants water soaked before cold storage were slightly (not significant) shorter and thicker than the post-storage water soaked and the operational no-water soaked treatments.

In Western hemlock plug seedlings, SIOS-treated plants were generally shorter than the controls (Fig. G9). Statistically significantly height reductions were found between the controls and SIOS treatments at -0.75 and -1.5 MPa before cold storage. Seedlings treated before cold storage at both concentrations were also significantly shorter than the post-treated seedlings.

Western hemlock mini-plugs: The effect of SIOS on the Western hemlock mini-plugs were generally similar to the effects observed on the hemlock plugs (Fig. G7). The effect of SIOS concentration was significant in reducing plant height at the -0.75 MPa pre-storage treatment only. In general, treatments before and after cold-storage were similar.

G.4.2.4 Packability assessment

No statistical differences were found between the controls in both species (Fig. G10). In Douglas-fir, the percentage packability between treatments was statistically not significant when comparison is made between the time of treatment of the controls and sorbitol concentrations. Statistically significant differences occurred between the operational controls and treatments of SIOS made before cold storage and the 10% post-storage treatment. In Western hemlock, the sorbitol treatment at -1.5 MPa before cold-storage had a dramatic effect on percent packability. No differences in percent packability were found when hemlock seedlings were treated at either concentrations after cold-storage.

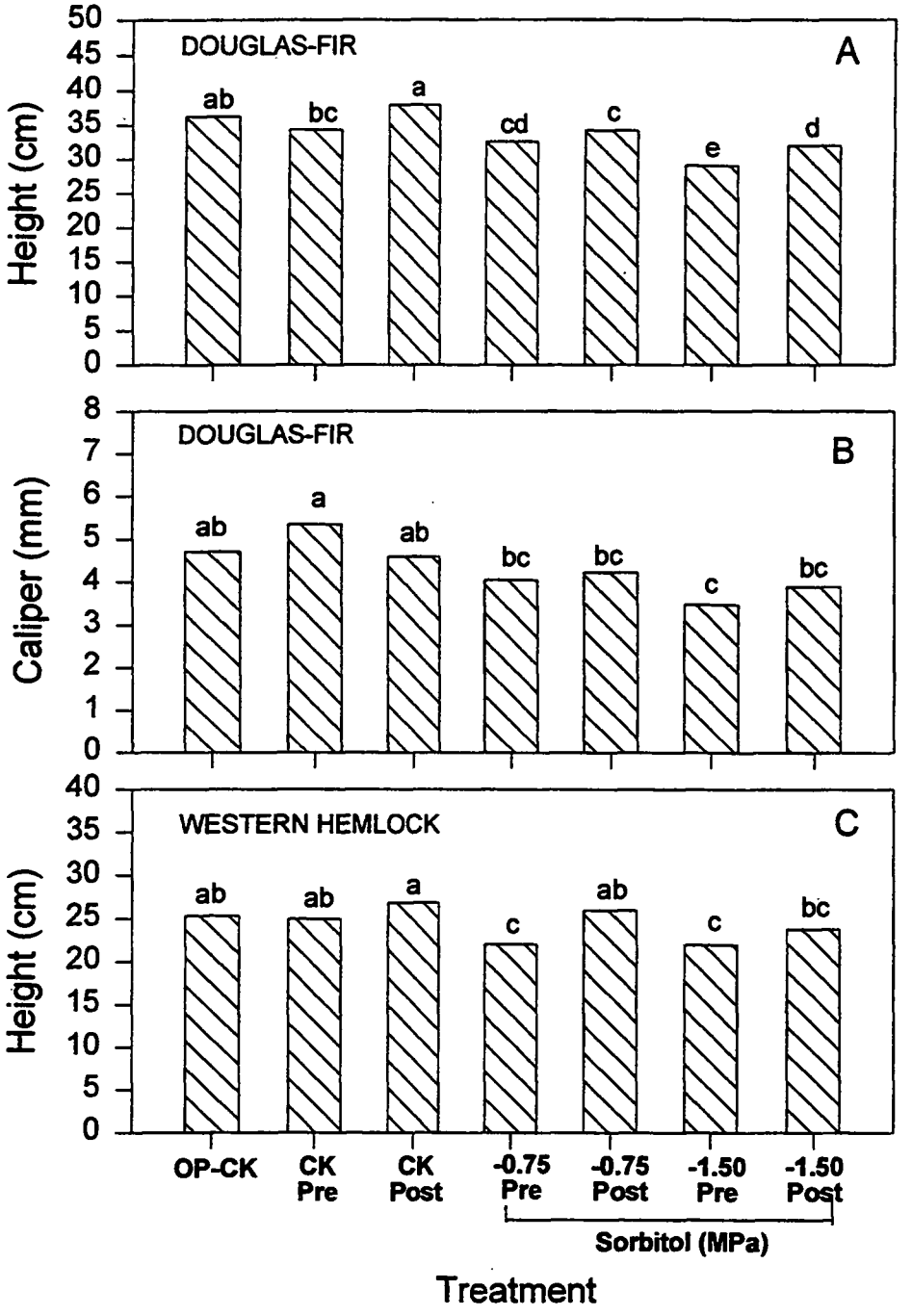

Figure G9. Mid-season assessment of plant height and stem caliper, approximately 13 weeks after transplanting at Mima Nursery, from Douglas-fir and Western hemlock seedlings treated before or after cold storage with either water, -0.75, or -1.5 MPa of sorbitol solution and transplanted on May 1, 1995 approximately 15 weeks after cold storage.

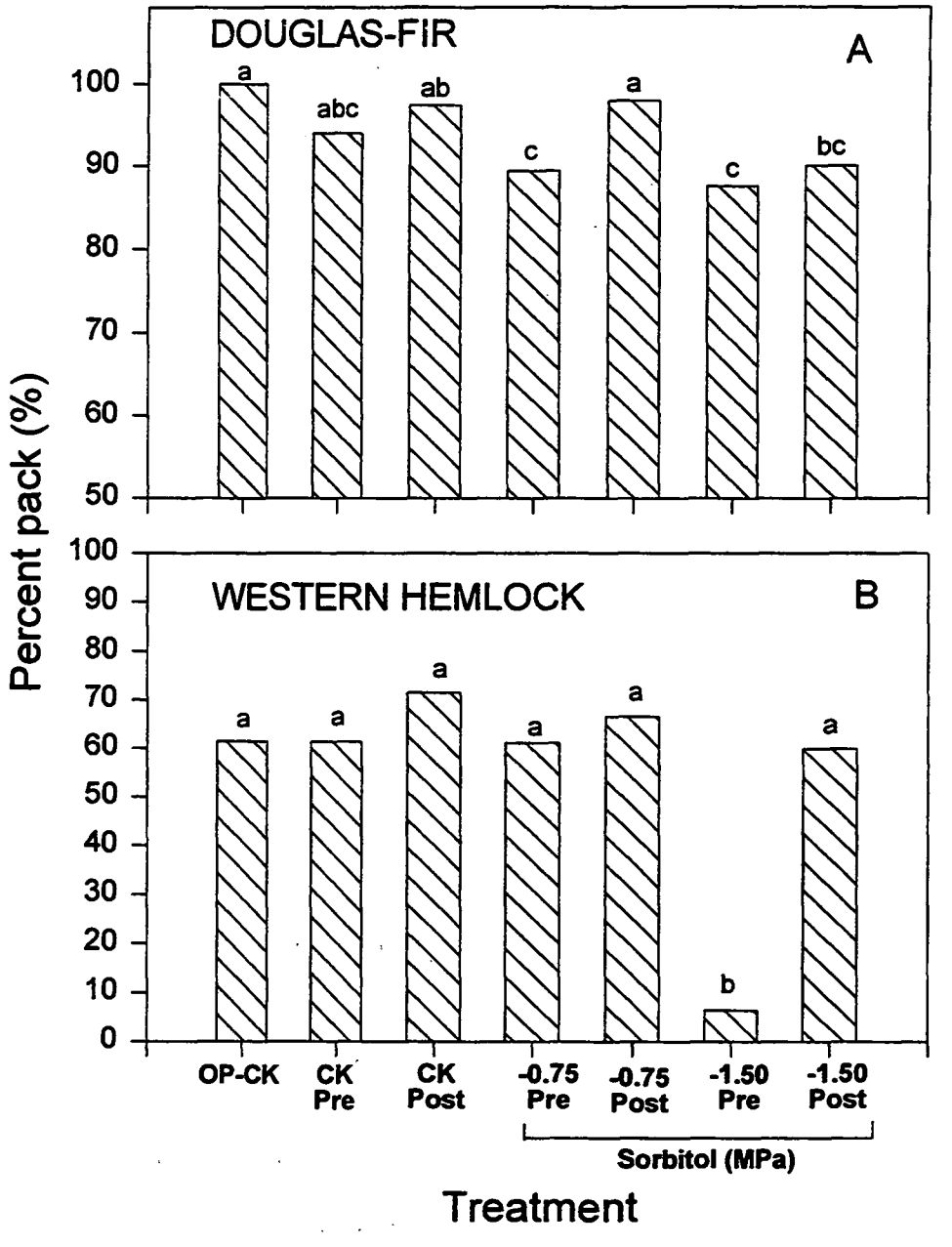

Figure G10. Packability of Douglas-fir and Western hemlock plug seedlings assessed approximately 32 weeks after transplanting at Mima Nursery. Seedlings were treated before or after cold storage with either water, -0.75, or -1.5 MPa of sorbitol solution, transplanted on May 1, 1995 at Mima Nursery, approximately 15 weeks after cold storage and grown up to the assessment on December 15, 1995. Operational control (OP-CK) is 'no water-soak-plants planted directly from cold storage'.

Table G4. Cold hardiness of needles of Douglas-fir determined 5 and 10 days after treatment with either water, -0.6, or -1.2 MPa of sorbitol solution (for details of treatments, see 'Cold Hardiness Test' in the section of Materials and Methods).

[z] A logistic sigmoid curve was fitted using all replicates of treatment to determine the temperature at 40% index of injury.

[y] A logistic sigmoid curve was fitted using a replicate to determine the temperature at 40% index of injury.

[x] Values in parentheses stand for freezing temperatures used for the freezing test of needles sampled 10 days after SIOS treatment.

[w] Values stand for (standard error) of percent index of injury determined at each freezing temperature.

Table G4.

Sampling Time	Treatment	Freezing temperature (°C) -5	-11	-17	-23(20)[x]	-26	-32(29)	Temp[y] (°C) at 40% Index of Injury	Temp[z] (°C) at 40% Index of Injury (Data pooled)
	CK	0.0	0.0	0.0	22.2 (9.0)[w]	35.7 (6.0)	45.8 (9.1)	-25.96	
	CK	0.0	0.0	0.0	13.3 (5.4)	18.8 (4.3)	58.3 (7.8)	-29.49	
	CK	0.0	0.0	4.2 (2.9)	15.7 (5.1)	31.3 (6.6)	64.4 (9.5)	-27.55	
							Mean (SD)	**-28.00 (1.32)**	**-28.03**
5 days after sorbitol Treatment	Sorbitol (-0.6 MPa)	0.0	0.0	0.0	10.4 (4.0)	35.7 (6.5)	64.7 (7.3)	-26.40	
	Sorbitol (-0.6 MPa)	0.0	0.0	0.0	8.3 (4.8)	37.8 (9.7)	60.0 (9.3)	-26.18	
	Sorbitol (-0.6 MPa)	0.0	0.0	2.1 (2.1)	37.8 (7.2)	37.8 (8.5)	70.8 (10.0)	-25.11	
							Mean (SD)	**-25.90 (0.69)**	**-26.32**
	Sorbitol (-1.2 MPa)	0.0	0.0	0.0	10.4 (4.0)	35.7 (6.5)	73.8 (10.0)	-26.36	
	Sorbitol (-1.2 MPa)	0.0	0.0	0.0	10.4 (4.0)	22.2 (5.3)	42.2 (7.6)	-30.87	
	Sorbitol (-1.2 MPa)	0.0	0.0	0.0	6.3 (3.4)	26.7 (6.7)	82.2 (7.9)	-27.08	
							Mean (SD)	**-28.10 (2.42)**	**-27.29**
	CK	0.0	0.0	3.0 (3.0)	6.1 (4.1)	46.2 (11.0)	51.3 (12.9)	-24.81	
	CK	0.0	0.0	11.1 (4.2)	20.0 (7.8)	42.2 (8.0)	91.1 (5.9)	-24.72	
	CK	0.0	0.0	8.9 (3.8)	40.7 (10.8)	63.3 (10.5)	83.3 (7.8)	-20.16	
							Mean (SD)	**-23.23 (2.66)**	**-24.25**
10 days after sorbitol treatment	Sorbitol (-0.6 MPa)	0.0	0.0	9.1 (4.7)	26.7 (10.9)	51.3 (13.4)	56.4 (12.7)	-22.14	
	Sorbitol (-0.6 MPa)	0.0	0.0	24.2 (5.9)	38.5 (11.8)	35.3 (10.5)	79.5 (8.9)	-19.51	
	Sorbitol (-0.6 MPa)	0.0	0.0	0.0	19.7 (3.79)	30.4 (4.09)	46.0 (2.68)	-23.31	
							Mean (SD)	**-21.65 (1.95)**	**-20.92**
	Sorbitol (-1.2 MPa)	0.0	0.0	15.4 (4.8)	20.0 (10.2)	66.7 (8.1)	79.2 (9.1)	-22.46	
	Sorbitol (-1.2 MPa)	0.0	0.0	19.6 (4.1)	48.9 (9.4)	69.1 (8.9)	84.4 (8.5)	-19.22	
	Sorbitol (-1.2 MPa)	0.0	0.0	12.5 (4.2)	42.2 (8.6)	66.7 (8.6)	77.9 (10.1)	-19.93	
							Mean (SD)	**-20.54 (1.70)**	**-20.72**

G.4.2.5 Cold hardiness studies

In three separate studies, SIOS had no significant effect on promoting or maintaining cold hardiness of Douglas-fir seedlings (Table G4). Plants treated with SIOS were slightly less hardy than the controls.

G.5 Conclusions

The sorbitol-induced osmotic stress was effective in reducing water loss of Douglas-fir and Western hemlock seedlings in greenhouse desiccation tests at Oregon State University and Weyerhaeuser Co. The effect of SIOS treatment either before or after cold-storage on reducing water loss in the greenhouse desiccation test was similar. In the greenhouse desiccation tests, the recovery and root growth potential of the treated plants were quite apparent (e.g. the controls all died and high percentages of the SIOS-treated plants survived). A higher percentage of the Douglas-fir compared to the Western hemlock seedlings survived the desiccation treatment.

In field tests, performed at the Weyerhaeuser Mima Nursery, SIOS-treated Douglas-fir and Western hemlock seedlings broke buds slower and were generally smaller than the controls. The plants treated with -1.5 MPa of sorbitol suffered more dieback and/or had less vigor than the -0.75 MPa treatment and controls. The percent of packabilty of plants was less in the sorbitol treatment at -1.5 MPa before cold-storage in both species. The sorbitol treatment at -1.5 MPa after cold-storage also decreased packability slightly in Douglas-fir but not Western hemlock.

The SIOS had no effect on cold acclimation of Douglas-fir seedlings.

APPENDIX H

REDUCING WATER LOSS OF CONTAINERIZED WHITE SPRUCE (*Picea glauca* Voss.)

Containerized 3-year-old white spruce (*Picea glauca* Voss.) were treated with either water, -0.75, -1.1, or -1.5 MPa of sorbitol solutions at a rate of 500 ml per gallon pot in a greenhouse set at 25°/18°C (day/night) with natural light on 25 February, 1994. Thereafter, water was withheld for 18 days. Plants were then rehydrated by being soaked in water for 3 min and thereafter well-watered.

During desiccation stress period, relative weight change in plant and medium was determined, and the relative tissue water content was measured from the shoot tip (approximately 5 cm-long) 20 days after water stress by relative tissue water content (%) = (tissue fresh wt – tissue dry wt)/(tissue initial wt – tissue dry wt) x 100. In addition, the percent plant recovery was determined 25 days after rewatering by scoring the overall color of needles based on the following recovery index (e.g., 1=almost brown, 2=moderately brown, 3=moderately green, 4=almost green, and 5=perfectly green). All measurements had 10 replications.

In general, water loss out of the plant and medium during desiccation stress period was inversely proportional to the concentrations of sorbitol with no difference between -1.1 MPa and -1.5 MPa (Fig. H1). After 18 days of water stress, plants treated with sorbitol-induced osmotic stress (SIOS) had higher tissue water content in all concentrations than did water-stressed controls (Fig. H2). After rewatering, SIOS-treated plants recovered much better than did water-stressed controls (Fig. H3).

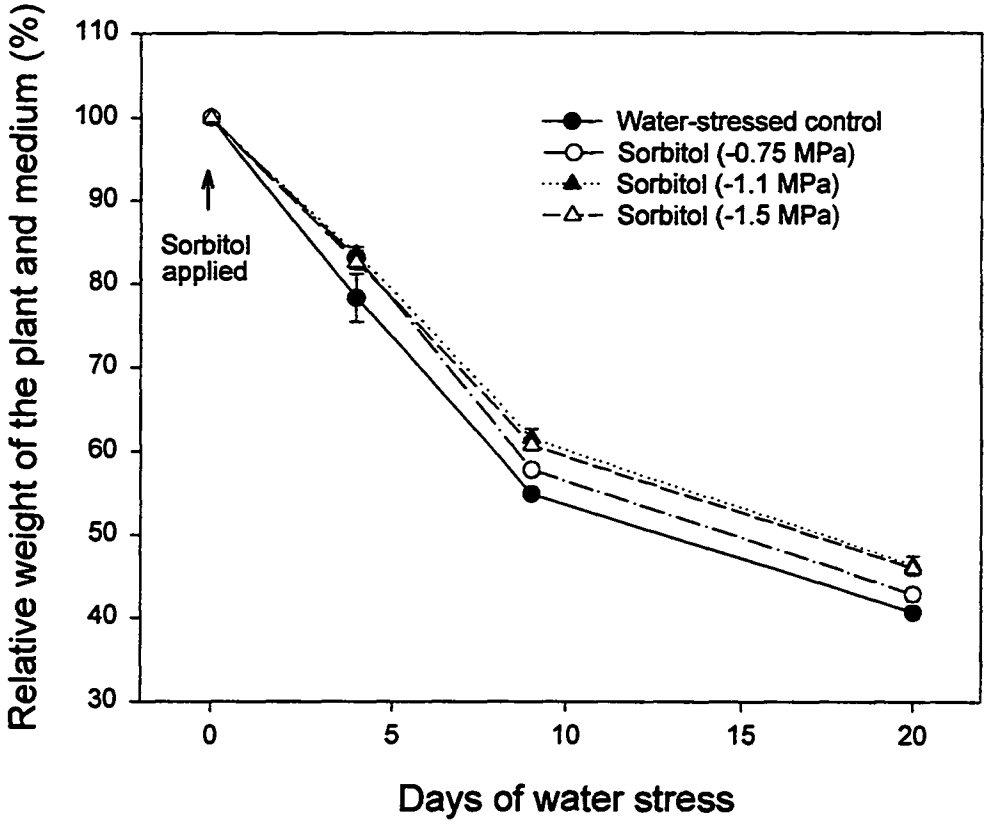

Figure H1. Relative weight change in the medium and white spruce treated with either water, -0.75, -1.1, or -1.5 MPa of sorbitol solution as a soil drench, with desiccation stress period.

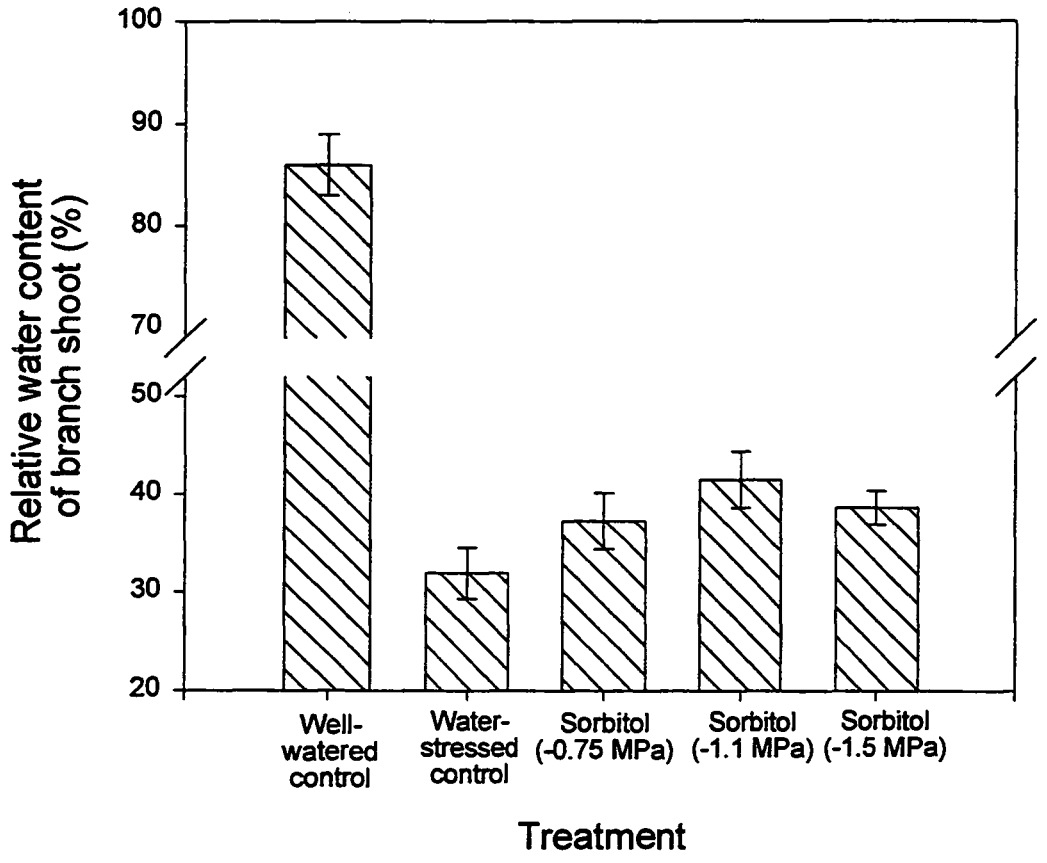

Figure H2. Relative water content of branch shoot in white spruce treated with either water, -0.75, -1.1, or -1.5 MPa of sorbitol solution as a soil drench, measured 18 days after desiccation stress.

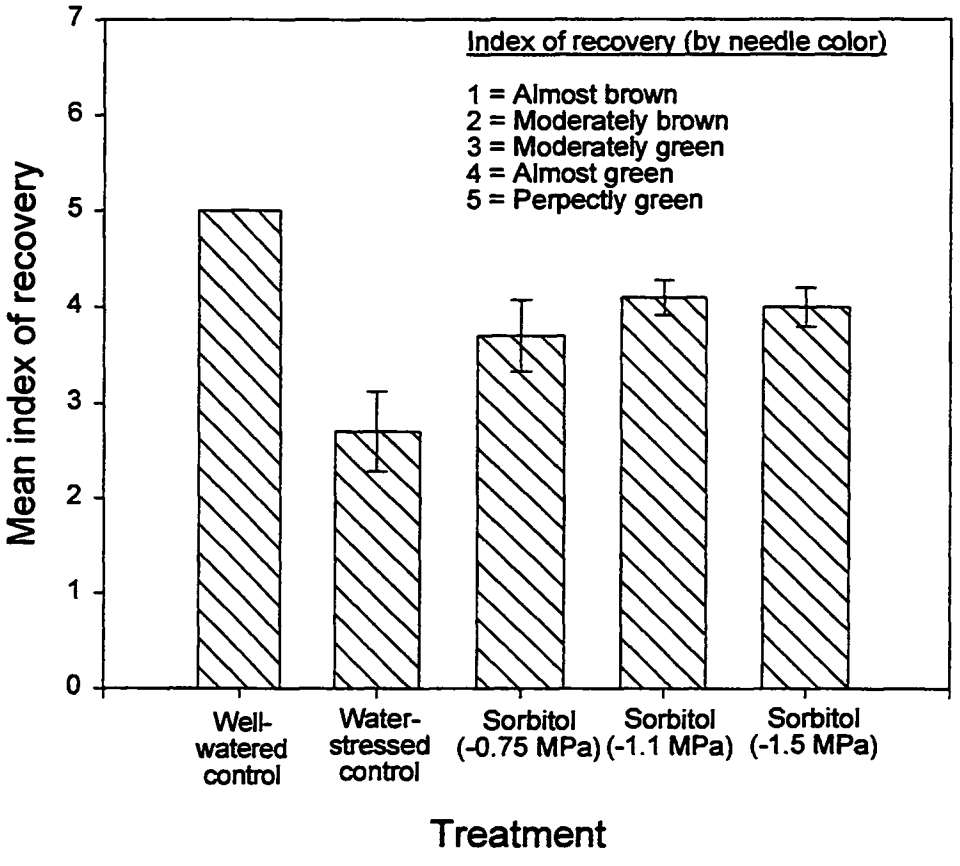

Figure H3. Mean index of recovery of white spruce treated with either water, -0.75, -1.1, or -1.5 MPa of sorbitol solution as a soil drench, exposed to 18 days of desiccation stress, and then rewatered. The index of recovery of plants was determined by scoring the color of needles 25 days after rewatering, based on 5 levels of indications shown in the figure.

Figure H4. White spruce recovered 25 days after rewatering, treated with either water, -0.75, -1.1, or -1.5 MPa of sorbitol solution as a soil drench, exposed to 18 days of desiccation stress, and then rewatered.

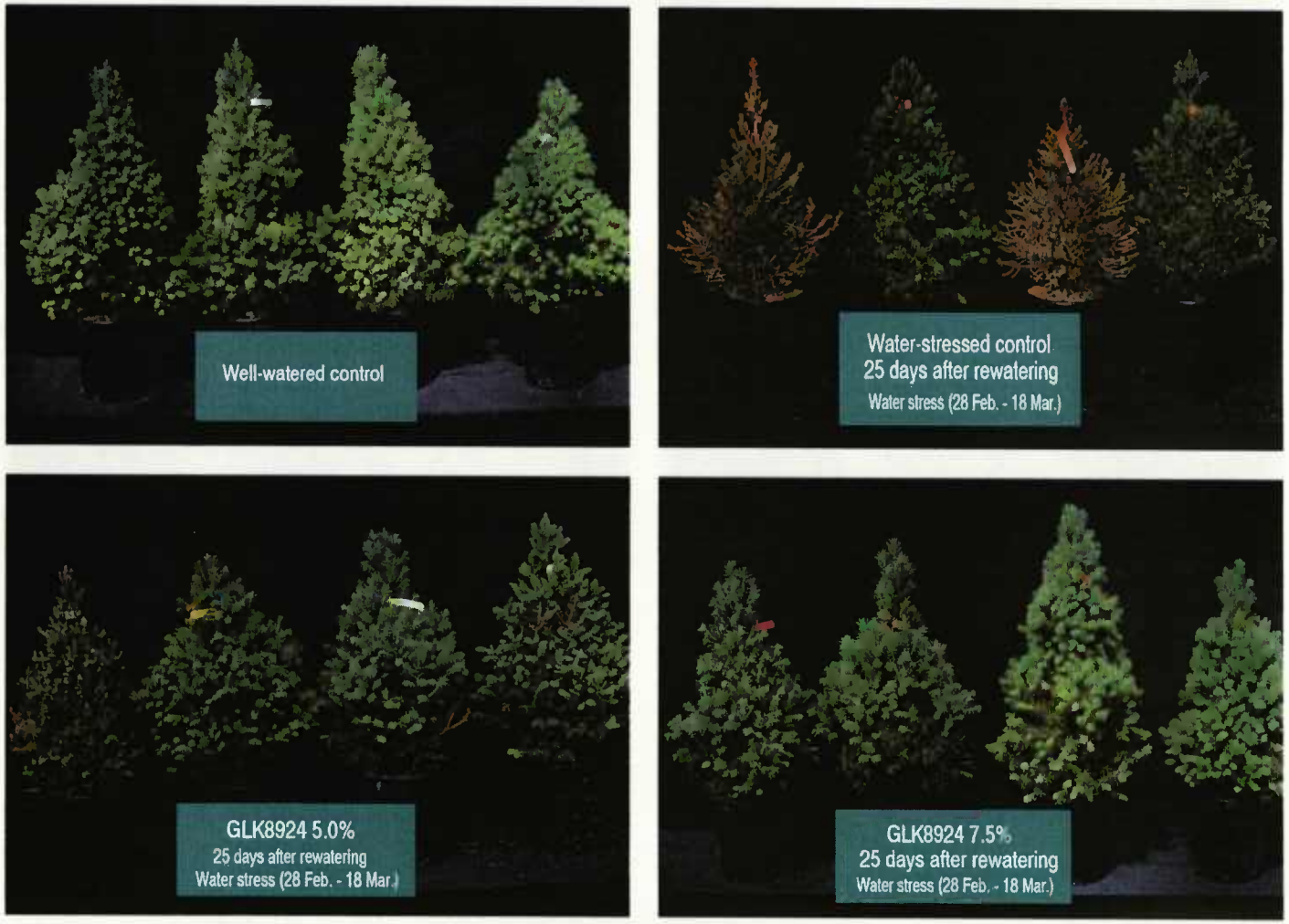
Well-watered control
Water-stressed control
25 days after rewatering
Water stress (28 Feb. - 18 Mar.)
GLK8924 5.0%
25 days after rewatering
Water stress (28 Feb. - 18 Mar.)
GLK8924 7.5%
25 days after rewatering
Water stress (28 Feb. - 18 Mar.)

Figure H4

APPENDIX I

EFFECT OF SORBITOL-INDUCED OSMOTIC STRESS ON REDUCING WATER LOSS IN CELERY, ROSE, LETTUCE, AND GARDENIA

Figure I1: Celery seedlings (A), mini-rose (B), lettuce (C), and gardenia (D) treated with either water or sorbitol as a soil drench and then exposed to water stress for 5 (A and C) and 7 (B and D) days.

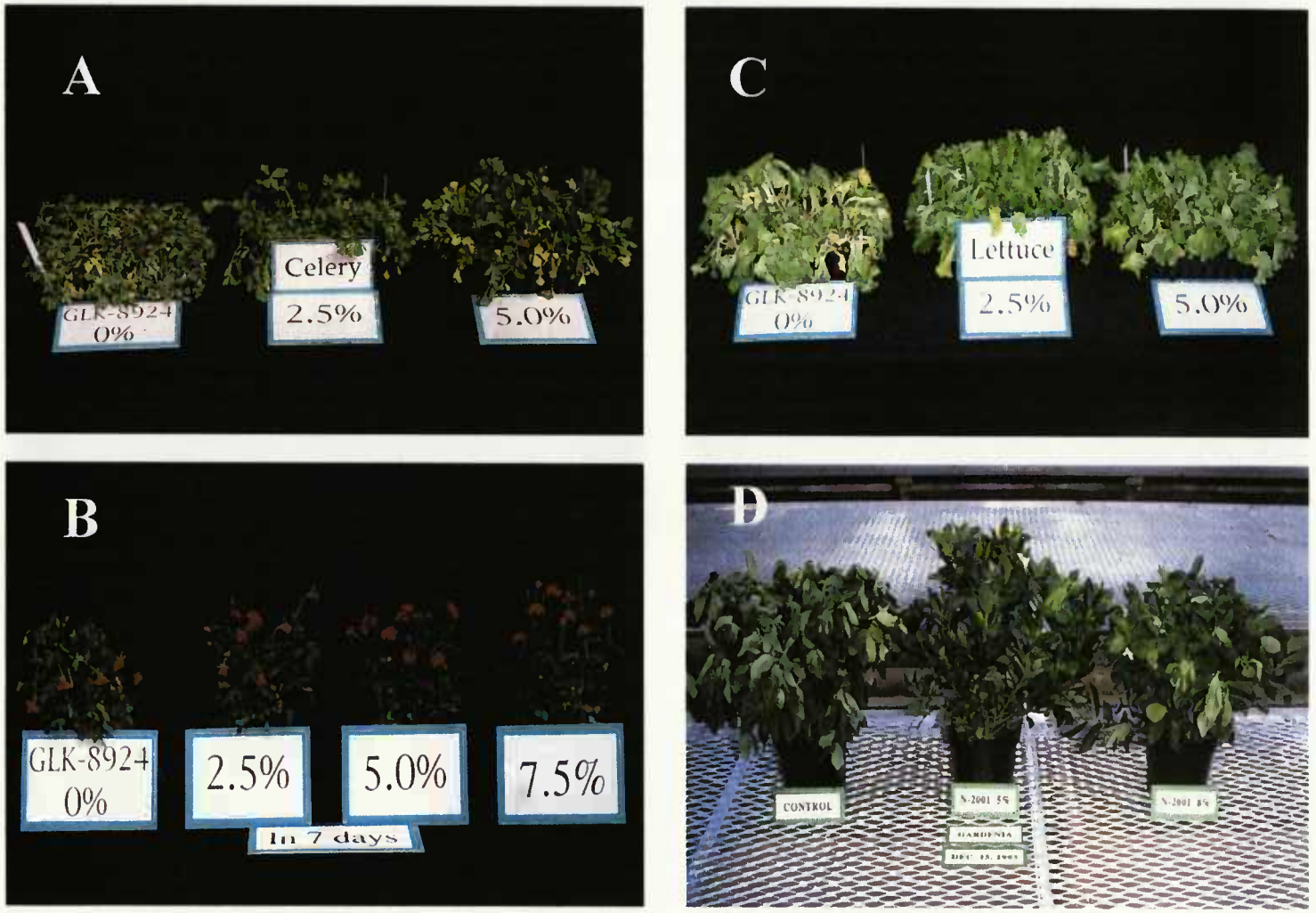
A
Celery
GLK-8924
0%
2.5%
5.0%
C
Lettuce
GLK-8924
0%
2.5%
5.0%
B
GLK-8924
0%
2.5%
5.0%
7.5%
In 7 days
D
CONTROL

Figure I1