AN ABSTRACT OF THE THESIS OF

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Patrick J. Breen

Identification of factors that affect water and sugar transport in strawberry could aid in attempts to influence fruit size and quality. Apoplastic water potential (ψ_{aw}) gradients within the strawberry plant, that could influence water and assimilate transport, were identified. Fruit expansion was very sensitive to water stress and occurred when the ψ_{aw} of leaves was higher than that of fruit. Green-white fruit underwent osmotic adjustment in drought stressed plants, but not to levels to where expansion could be maintained during daytime.

Solutes in the apoplast of developing strawberry fruit may contribute significantly to a ψ_{aw} gradient within in the plant. We utilized four techniques to determine fruit apoplastic solute potential (ψ_{as}). First, ψ_{as} was estimated from the difference between ψ_{aw} (measured via psychrometry) and the xylem water potential (ψ_x) (measured with a pressure chamber); secondly, xylem exudate was collected via a pressure chamber; thirdly, liquid was recovered by centrifugation of plugs of fruit tissue; and finally, apoplastic solution was collected on paper disks by a novel method developed to sample the open pith cavity of ripening strawberry fruit with little or no cell damage. All methods, except the xylem

exudate method, suggested moderate levels of apoplastic solutes (more negative ψ_{as}) in ripening fruit, including concentrations of sucrose and glucose each near 50 mM.

Turgor-sensitive sugar uptake from the apoplast of sink tissues may represent a point of regulation in translocation of sucrose by influencing the rate of uptake of sucrose from the sink apoplast. To determine whether ψ_{aw} of bathing solutions (and thereby cell turgor) influenced sugar uptake by fruit cortex tissue from the apoplast, disks were cut from green-white fruit and placed into incubation solutions at various osmolarities. Lowering cell turgor decreased *in vitro* uptake of C^{14}-sucrose and C^{14}-glucose by tissue disks. Strawberry fruit cells apparently do not possess a sugar uptake system that is stimulated by a reduction in turgor, as found in some plants.

Expansion, Apoplastic Solutes, and Sugar Uptake in Developing Strawberry Fruit.

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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.

Kirk W. Pomper, Author

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CONTRIBUTION OF AUTHORS

Dr. Patrick Breen was involved in the experimental design, discussion and editing of successive drafts of each manuscript.

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CHAPTER 1

INTRODUCTION

About 90% of the total weight of a ripe strawberry (*Fragaria* x *ananassa* Duch.) fruit is water (Darrow, 1966), so the transport of water and assimilate is an important component in achieving optimum growth and development of the strawberry fruit. Major gains in agricultural crop yields have come through increased partitioning of assimilate from leaves (sources) to the harvestable storage organs (sinks) (Gifford et al., 1984). Identification of the pathways by which water and sugar are transported to strawberry fruit may allow their manipulation via breeding or biotechnological methods, increasing both yields and possibly fruit sugar levels. The water status of a plant organ can be represented by its water potential. Water moves in the xylem along gradients within the plant toward lower water potentials, with gradients created between organs and tissues due to differences in evaporational losses, solute accumulation, and the water demand needed for cell expansion (Nonami and Boyer, 1993). However, xylem is not the only pathway by which water is transported within the plant, water is in fact the major substance translocated in the phloem. Because of physical disruption of the xylem, the phloem is often the dominant pathway of water transport into developing fruit (Lee, 1991). Diurnal fluctuation in expansion has been reported in many fruit (Berger and

Selles, 1993) and is related to the water potential gradient between the stem and fruit (Johnson et. al, 1992).

Even mild levels of drought stress can greatly reduce overall strawberry fruit expansion (Renquist and Breen, 1982; Dwyer et al., 1987). Since water potential = turgor potential + solute potential, when turgor potential is unchanged, a decrease in solute potential lowers the water potential of a plant organ. Osmotic adjustment (the active accumulation of solutes by cells) can decrease solute potential and thereby maintain positive turgor under drought stress, allowing continued cell expansion and metabolic functions that require positive turgor (Morgan, 1984). Larger water potential differences between tissues enhances water and assimilate transport toward the tissue with lower water potential. Strawberry leaves undergo osmotic adjustment with drought stress (O'Neil, 1983). The reductions in fruit expansion under drought stress may indicate that these tissues fail to osmotically adjust to levels to achieve sufficient turgor for cell expansion.

The rate of transport of assimilate via the phloem into sink regions such as strawberry fruit may be influenced by solutes in the extracellular space or apoplast (the whole continuity outside the plasma membrane). High levels of apoplastic solutes would be expected to promote the removal of water from the phloem, reducing its turgor, and presumably that of sink cells, and increase the efflux of translocated solutes into the apoplast for subsequent uptake by sink cells (Lang and Thorpe, 1986). Phloem transport to a sink organ has been enhanced by lowering its water potential through incubation in a non-permeating osmoticum, demonstrating that high levels of apoplastic solutes can

influence translocation (Lang and Thorpe, 1986). Continued transport of sugars and other solutes to sink regions containing high apoplastic solutes could be maintained if declining turgor stimulates solute uptake by sink cells. Turgor-sensitive uptake has been observed in many sink tissues, where cell turgor of tissue disks is reduced in incubation solutions containing non-permeating osmoticum, resulting in increased uptake of exogenously supplied sugar (Wyse et al., 1986; Oparka and Wright, 1988a and 1988b). Apoplastic water would move into sink cells as they accumulated solutes, allowing continued water and assimilate import in to the region. Neither the levels of solutes in the fruit apoplast, nor turgor-sensitive uptake by strawberry fruit cells has been examined.

Sucrose, the major sugar translocated in strawberry (Forney and Breen, 1985a), could be unloaded via a symplastic route (via plasmodesmata) as well as into the apoplast prior to its uptake into fruit cells (Forney and Breen, 1986). An improved understanding of the mechanisms of water and sugar transport could help identify strategies to increase transport to the fruit and increase its expansion and sugar accumulation under water-stress. The objectives of this thesis research were to: a) relate plant water relations to fruit expansion during water-stress, b) to assess the contribution of apoplastic solutes to fruit water potential, and c) determine whether the uptake of sugars by fruit cells from the apoplast was turgor-sensitive.

CHAPTER 2

LITERATURE REVIEW

2.1 Strawberry fruit morphology and growth

The strawberry fruit is an aggregate fruit with achenes (these small, dry one-seeded organs are the true botanical fruit) on the surface of a fleshy receptacle. The receptacle is composed of: a) an epidermis, made up of polygonal cells, b) a hypodermis, consisting of meristematic cells, c) a cortex composed of rounded parenchyma cells, comprising most of the fleshy portion of the fruit, d) a bundle zone, the ring of vascular tissue with individual branches leading to each achene, and e) pith, consisting of thin-walled cells which often separate during fruit growth leaving large cavities (Winton and Winton, 1935).

The strawberry fruit is borne on a type of inflorescence known as a cyme which gives rise to a hierarchy of fruit; the largest is in the primary (1^{o}) position, with succeeding smaller fruit at lower ranks designated as secondary (2^{o}), tertiary (3^{o}), quaternary (4^{o}), etc. In addition to position within an inflorescence, fruit size is also dependent upon the number of developed achenes (Janick and Eggert, 1968), the number of cells (Cheng and Breen, 1992), fruit competition, and plant vigor (Darrow, 1966; Janick and Eggert, 1968). The growth of the strawberry fruit, as measured by an increase in weight or volume, often shows a sigmoidal pattern (Knee et al., 1977, Woodward, 1972), although a double sigmoidal growth curve has also been reported (Perkins-Veazie

and Huber, 1987; Miura et al., 1990). In a study of three cultivars that vary in final fruit size, Cheng and Breen (1992) found that the relative difference in cell number per fruit is established by anthesis, cell division continued until about 12 days after anthesis (DAA), and mean cell size of mature fruit cells was similar in all cultivars.

Strawberry fruit expansion is very sensitive to drought, with even mild water stress greatly reducing yields (Renquist, 1982; Dwyer et al., 1987). Even under favorable environmental conditions few strawberry fruit expand to their fullest potential (Abbott et al., 1970). Water deprivation limits cell expansion and fruit growth, and Webb (1973) suggested that limitations in the water supply capacity of the strawberry peduncle/pedicel may prevent most fruit on a single inflorescence from attaining full size.

2.2 Fruit expansion and plant water potential

ψ_w is the chemical potential of water expressed in units of pressure (usually MPa) (Nobel, 1983). The water status of a plant organ can be described by its water potential (ψ_w), which is considered the sum of the solute potential (ψ_s) and the turgor potential (ψ_p), when the gravitational component is ignored. Water moves within the plant along decreasing gradients in ψ_w. These gradients are created between plant organs and tissues by differences in evaporation rates, solute content, and cell expansion (Nonami and Boyer, 1993). Diurnal fluctuation of expansion has been reported in many fruit and is related to the difference in ψ_w between the stem and fruit (Johnson, 1992; Tromp, 1984; Berger and Selles, 1993). Lee (1989a) proposed that unidirectional water flux into

tomato fruit is driven by fruit growth and transpirational losses, creating a ψ_w gradient between the stem and fruit. Johnson et al. (1992) showed that there is a strong correlation between tomato fruit expansion and the ψ_w difference between the fruit and stem, with a ψ_w gradient favoring solution flow into fruit coinciding with a rapid increase in fruit diameter, whereas inverting the ψ_w gradient caused fruit shrinkage. Fruit shrinkage can result from a reversal in water flow, and water fluxes from apple fruit toward the trunk during water stress have been measured using a heat-pulse method (Yamamoto, 1983).

Evaporational loss from fruit likely plays only a limited role in influencing fruit ψ_w and expansion. Lee (1990) suggested that fruit transpiration in tomato may exceed the delivery capacity of the conducting tissues in the pedicel causing fruit shrinkage. However, Johnson (1992) found that only a small fraction (10.5 %) of the water imported into tomato fruit is lost to transpiration. Wrapping of apple fruit in foil to restrict transpiration only reduces diurnal fruit shrinkage by 20-35% and had little influence on fruit ψ_w (Tromp, 1984).

2.3 Osmotic adjustment

Osmotic adjustment, the maintenance of positive cell turgor through the accumulation of solutes within cells, is often a strategy by which plants tolerate salt or water stress (Morgan, 1984; Kramer and Boyer, 1995). Positive cell turgor is required for plant growth (Meyer and Boyer, 1981), opening of stomata, photosynthesis, and nitrogen metabolism (Kramer, 1983; Turner and Jones, 1980). Cell turgor is equal to ψ_w minus

the ψ_s (i.e. $\psi_p = \psi_w - \psi_s$). The accumulation of solutes in cells can lower ψ_s, thereby increasing ψ_p, allowing turgor dependant processes to continue at progressively lower leaf ψ_w. If ψ_p and cell wall yield (the minimum turgor below which no expansion occurs) remain steady, reductions in ψ_s reduce ψ_w of cells (i.e. $\psi_w = \psi_p + \psi_s$), and may increase water and assimilate transport toward tissues with reduced ψ_w (Cosgrove, 1993). Jones et al. (1985) have suggested that osmotic adjustment likely occurs in fruit tissues, but this has not been reported in the literature.

Osmotic adjustment occurs in the leaves of *Fragaria* x *ananassa* Duch. (Renquist et al., 1982), *F. chiloensis* (Archbold and Zhang, 1991), and *F. virginiana* (O'Neill, 1983) in response to drought stress. Archbold and Zhang (1991) have suggested that the greater degree of osmotic adjustment shown in *F. chiloensis* than in *F. virginiana* could contribute to better growth and survival of *F. chiloensis* under drought. It has not been determined whether the fruit of strawberry under drought are capable of osmotic adjustment as a means to maintain growth and influence plant ψ_w gradients so as to effect water transport.

2.4 Measurement of water potential

The ψ_w of a plant organ is usually measured either via psychrometry or a pressure chamber. With the pressure chamber method, a plant organ is severed and placed into a chamber where the pressure is increased until fluid, presumably from the xylem, is pushed from the cut end. This pressure, although opposite in sign, is considered to be

numerically equivlant to the ψ_w of the organ; assuming solute levels in the xylem are very low (i.e. $\psi_w = \psi_p$). Obviously one major drawback of the pressure chamber method is that the destructive sampling prevents repeated measurements of the same organ.

The validity of ψ_w measurements made with a pressure chamber to represent the total water potential of a tissue has been called into question. Xylem tension measured with a pressure probe is much lower than that determined with a pressure chamber (Balling and Zimmermann, 1990; Zimmermann et al., 1993; Zimmermann et al., 1994; Benkert et al., 1995). Using NMR-microscopy, Zimmermann et al. (1993) reported that drought stressed leaves show large amounts of air in the tissue and they suggest that air-filled spaces interfere with the propagation of pressure across the tissue in the pressure chamber, leading to overly negative ψ_w measurements. High resolution ^{1}H-NMR-imaging of tissue a few millimeters from the cut ends of excised tobacco leaves also indicate that xylem water does not recede from the cut end as has been assumed for many years (Zimmermann et al., 1993).

In psychrometry, either plant tissue is placed in a chamber containing a thermocouple or the chamber apparatus is place on an organ while still attached to a plant. After a period of time, the water vapor in the chamber comes into equilibrium with the ψ_w of the tissue. There is a thermocouple junction inside psychrometer chamber which is then cooled to a temperature below the dew point of the air of the chamber by passage of an electrical current that causes water to condense on the junction, then the current is discontinued. Water on the junction evaporates, cooling it, with the magnitude of the temperature depression dependant on the humidity (water vapor pressure) of the

surrounding air. The temperature difference before and after evaporative cooling of the junction is directly related to the relative humidity and hence ψ_w (see Appendix B for further information of the theory and operation of a psychrometer). Measurement of the ψ_w of a given leaf with psychometric and pressure chamber methods yield similar values. Since measurement of ψ_w of plant tissue with a pressure chamber may not represent the true ψ_w, it causes one to wonder whether psychrometers also yield accurate measurements of ψ_w of organs.

2.5 Phloem loading and unloading

Sucrose and other components of the phloem sieve elements are translocated by means of a passive bulk flow down a gradient of hydrostatic pressure (Giaquinta, 1983). Sink regions (areas where carbon is utilized) apparently influence the direction and magnitude of translocation by locally removing water and solutes from the sieve tubes, thus steepening the osmotic and chemical gradient between source leaves and sink tissues (Thorne, 1986). The bulk flow of solutes in the phloem also results in the transport of significant amounts of water to sink regions.

Excellent reviews of carbon partitioning and phloem loading can be found in Frommer and Sonnewald (1995) and Wardlaw (1990). The path of transfer of sucrose from its site of synthesis to the leaf phloem depends on the plant species, but can consist of an apoplastic (the whole continuity outside the plasma membrane) (e.g. sugar beet, potato, etc.) or a symplastic (via plasmodesmata) route (e.g. cucumber) (Wardlaw, 1992).

Significant advances have been made recently in understanding phloem loading which deserve mention. Molecular approaches have led to the cloning of a sucrose transporter that is thought to be located in the sieve element plasma membrane, and is involved in phloem loading from the apoplast in potato (see the review of Bush, 1993). Yeast are able to use sucrose as a sole carbon source by secreting invertase (which hydrolyses sucrose to its hexose meioties) and subsequently transporting the released hexoses into the cell. Some strains are also able transport sucrose through a maltose transporter. Riesmeier et al. (1992) isolated a yeast mutant deficient in both invertase activity and maltose transport. After confirming that cells of this strain could not grow on sucrose, they re-introduced cytosolic invertase or sucrose synthase (neither are secreted) to allow these mutants to metabolize sucrose, but only after it enters the cell. These yeast mutants were transformed with a cDNA library from spinach leaves, which presumably contained the gene for the sucrose transporter, constructed in a yeast expression vector and then screened for transformants that restore growth on sucrose. Growth on sucrose would indicate that either the mutant yeast had acquired sucrose transport or secreted invertase activity. Riesmeier et al. (1992) identified a clone that encoded for a sucrose transporter. This group went on to show that this sucrose transporter plays an essential role in phloem loading and assimilate partitioning in potato using antisense technology (Riesmeier et al., 1994). H^+ATPase genes have also been cloned from potato (Harms et al., 1994) and could be important in phloem loading since sucrose transporters are sucrose-H^+ symporters. Gahrtz et al. (1994) have also cloned a phloem-specific sucrose transporter from *Plantago major* L. supporting the model of apoplastic phloem loading.

Several reviews on phloem unloading have been published recently, so it will only be outlined briefly here along with some recent significant contributions in this area (see Oparka, 1990; Patrick, 1990; Wardlaw, 1990; Wolswinkle, 1990; Thorne, 1986). Several processes may serve to promote continued unloading from the phloem into sink regions: 1) chemical alteration of the unloaded assimilates (e.g. hydrolysis of sucrose to glucose and fructose, or formation of starch), 2) compartmentation within apoplastic or symplastic pools, or, 3) utilization of assimilates for growth (Thorne, 1985).

Unloading of sugars from the phloem may occur through the symplast or apoplast or a combination of both (Oparka, 1990; Patrick, 1990). The unloading of solutes from the phloem may involve an active transport step (Gifford and Thorne, 1986), but evidence that unloading is passive mechanism has also been reported (Porter, 1985). It is not currently known whether the sucrose transporter recently cloned from the phloem is involved in unloading, such as backflow through the sucrose transporter (Riesmeier et al., 1992; Frommer and Sonnewald, 1995). Extensive plasmodesmata connections from between the phloem and surrounding tissues, as demonstrated in ultrastructural studies and by the movement of fluorescent probes, have been used as evidence for a symplastic route. This route is the major pathway of unloading in expanding leaves (Turgeon, 1989), root apices (Giaquinta et al., 1983) maternal tissues of developing seeds (Thorne, 1985), and potato tubers (Oparka 1986; Oparka and Prior, 1987, 1988).

Common features of sinks in which phloem is unloaded symplastically are metabolism of imported photosynthate that is based on polymer formation (e.g. starch) and sink cells with turgor less than that of the sieve elements (Patrick, 1990). Apoplastic

pathways of unloading are thought to occur in sugar cane stems (Glasziou and Gayler, 1972), sugar beet tap roots (Wyse, 1972), and citrus (Koch et al., 1986) and tomato (Damon et al., 1988) fruits. These sinks tend to accumulate osmotically active solutes to high concentrations (Patrick, 1990). A symplastic unloading route may be important early in the development of some fruit (e.g. tomato), with apoplastic unloading becoming the major route later in development (Patrick, 1990).

A great deal of our knowledge of phloem unloading is based on solute release from the maternal tissues of legumes using the "empty seed coat" system (Thorne, 1985; Wolswinkle, 1990, 1992; Bradford, 1994). Recently, Wang and Fisher (1993, 1994) have elegantly examined the symplastic and apoplastic steps of phloem transport into developing wheat grains using fluorescent probes (e.g. Lucifer yellow). These authors also used microautoradiographic analysis of kernel sections and collected phloem exudate via aphid stylets after pulse labeling the plant with $^{14}CO_2$ to examine transport and unloading of ^{14}C-sucrose.

Recent work by Lucas et al. (1993) have changed the view of plasmodesmata as passive cytoplasmic bridges between neighboring cells to that of a supramolecular complexes consisting of membranes and proteins. They provide evidence that the plasmodesmata complex controls the size exclusion limit for intercellular diffusion of metabolites and small molecules as well as regulates the intercellular trafficking of macromolecules, including proteins and nucleic acids. Plasmodesmata may act as turgor regulated valves, controlling the outward movement of solutes from the sieve elements (Oparka and Prior, 1992).

The general inaccessibility and fragility of the phloem has hampered experiments concerning the unloading of solutes. A significant recent advance in examining phloem unloading has been the use of confocal laser scanning microscopy in the laboratory of K.J. Oparka, enabling real-time imaging of phloem unloading in the root tip of *Arabidopsis* seedlings (Oparka et al., 1994). The fluorescent probe 5(6) carboxyfluorescein (which is restricted to the symplast upon entry into phloem sieve elements due to its charged nature) was loaded into a single cotyledon and the entire seedling placed in a chamber for observation under the microscope. The probe was observed to be translocated rapidly to the root tip and unloaded from the phloem into discrete concentric files of cells. This unloading site corresponded to the position of two protophloem files of sieve elements, demonstrating a functional role of these cells in symplastic sieve element unloading. Oparka's group has shown that phloem transport processes can be imaged in real time and non-invasively within a intact plant, providing that the phloem is relatively close to the plant surface.

Molecular biological approaches to understanding phloem unloading have thus far been basically limited to alterations of sink cell metabolism. Müller-Röber et al. (1992) inhibited starch synthesis in transgenic potato by expressing a chimeric gene encoding antisense RNA for ADP-glucose pyrophosphorylase, which is thought to regulate starch synthesis. This reduced the activity of this enzyme and lowered starch contents to 2 to 5% of wild-type levels. In addition to inhibition of starch synthesis, the number of tubers and both fresh and dry tuber weight were reduced per plant, suggesting a reduction in sink strength. Stark et al. (1991) created transgenic potato plants in which ADP-glucose

pyrophosphorylase was over expressed only in the tuber, compared to wild type plants, and reported increased starch and higher dry matter content in tubers.

The function of phloem proteins (P-proteins) are incompletely understood but appear to be involved in plugging damaged phloem. Several cDNAs have been isolated that encode the expression of these proteins within companion cells in the vascular bundles of pumpkin (Bostwick et al., 1992). Phloem specific promoters have been identified (Martin et al., 1993) and will hopefully allow future manipulation of unloading pathways and alteration of metabolism within sieve elements.

2.6 Phloem transport to fruit

The phloem is a major pathway for water and solute movement into fruits. Water is also delivered via the xylem, but a break or discontinuity of the xylem has been reported late in fruit development in grape (Findlay et al., 1987), tomato (Lee, 1989), and apple (Lang and Ryan, 1994). The physical disruption of the xylem presumably limits its ability to transport water, and Lee (1989b) estimated that approximately 77% of the flow of water into a tomato fruit late in development takes place via the phloem.

Sucrose is the major sugar translocated in strawberry (Forney and Breen, 1985a) and may be unloaded into the fruit apoplast prior to uptake by fruit cells (Forney and Breen, 1986). Although a fruit-pedicel abscision zone does not form in strawberry, in many cultivars ripe fruit can be easily detached at the calyx, possibly reflecting a weakening in the vasculature in this region.

2.7 Apoplastic solutes and phloem transport

Solutes can be present at high levels in the apoplast of some sink tissues such as mature sugarcane stalks (Welbaum and Meinzer, 1990), developing seeds (Wolswinkle, 1990), tomato pericarp (Ruan et al., 1995), and ripening grape berries (Lang and During, 1991). The apoplast can serve as a storage area for sugar. Sucrose in the apoplast of stems of mature sugarcane may reach concentrations of 700 mM and account for up to 20% of the stored sugar (Welbaum and Meinzer, 1990). Apoplastic water potential (ψ_{aw}), which is considered to represent ψ_w of a tissue (Nobel, 1983), can be measured via psychrometry (Dixon and Tyree, 1984). High concentrations of apoplastic solutes in sink tissues may augment ψ_{aw} gradients within plants and influence water and assimilate flow from source to sink organs.

Lang and Thorpe (1986) have hypothesized that high levels of apoplastic solutes promote removal of water from the phloem, by reducing its turgor, causing increased efflux of translocated solutes into the apoplast for subsequent active uptake by sink cells. Apoplastic water would move into these cells due to increased solute content (i.e. reduced ψ_s), allowing continued water and assimilate transport to the region. These authors showed that steepening the ψ_{aw} gradient between the shoot and root of *Phaseolus vulgaris* L., by bathing the root in a solution containing a non-permeating osmoticum (mannitol), increased assimilate transport to the root. Solute levels in the apoplast of grape berries are relatively high and this may be the result of leakage from cells as fruit mature and senesce (Lang and During, 1991). High concentrations of apoplastic solutes

may be maintained because fruit transpiration is low, which results in reduced xylem flow to fruit which would increase dilution. The fruit pedicel of grape may essentially isolate apoplastic solutes from the dilute xylem solution in the stem due to its long diffusional path. Extreme swelling of cell walls and degeneration of plastids have been noted in ripening strawberry fruit (Knee et al., 1977), suggesting that a loss in cell membrane integrity could also result in a rise in apoplastic solute levels.

Direct quantification of solutes in the apoplast of plant tissues is technically very difficult. Washing-out (Glasziou and Gayler, 1972) or centrifugation of tissue segments (Terry and Bonner, 1980) have been used to obtain solute directly from the apoplast. However, a serious problem with these techniques is contamination of samples by the contents of cells damaged during the process (Ehwald et al., 1980). The pressure chamber has been used in attempts to sample the apoplast of leaves by collecting the xylem fluid pushed from the pedicel (Jachetta et al., 1986). Physical, semi-permeable barriers surrounding vascular bundles have been reported that could interfere with the free movement of apoplastic solutes, both in storage organs, such as sugarcane stalks (Welbaum, et al., 1992) and leaves (Canny, 1993), possibly preventing the retrieval of authentic apoplastic solution via the pressure chamber. Ruan et al. (1995) have successfully retrieved apoplastic solution under pressure through a syringe needle inserted into a tomato fruit using a pressure chamber method. The indirect estimation of apoplastic solutes levels can be calculated by assuming that the apoplastic solute potential (ψ_{as}) equals the difference between ψ_{aw} and $\psi_{x\text{-}press}$ (xylem pressure potential or tension) (i.e., $\psi_{as} = \psi_{aw} - \psi_{x\text{-}press}$).

2.8 Sugar uptake from the apoplast

Plant cells possess the capacity to accumulate solute supplied exogenously to the apoplast (see Oparka, 1990). Sugar uptake from the apoplast by sink cells may represent the final step of apoplastic unloading from the phloem. However, Maynard and Lucas (1982) have suggested that uptake of sugars from the apoplast by cells may represent a general mechanism by which cells retrieve leaked sugars.

Turgor-sensitive uptake has been observed in many sink tissues. Increasing the concentration of a non-permeating osmoticum bathing plant tissues, thus lowering cell turgor, increases uptake of exogenously supplied sucrose by tissues of sugar beet taproot (Wyse et al., 1986), potato tuber (Oparka and Wright, 1988a and 1988b), leaves (Daie and Wyse, 1985) and developing cotyledons of *Phaseolus* (Patrick, 1994a). Increased uptake of sucrose from the apoplast by sink cells triggered by reduced turgor could be a strategy by which sink regions promote continued import of assimilates. However, this tactic may not be used by all tissues, for reducing turgor failed to enhance sucrose uptake in disks of *Citrus sinensis* leaves (Martinez-Cortina and Sanz, 1994) and carrot root tissue (Hole and Dearman, 1994).

The uptake of sugars *in vitro* often yields biphasic uptake kinetics with a saturable (carrier mediated) component and a linear (diffusional) component (see Oparka, 1990; Bush, 1993). The saturable component of *in vitro* sucrose uptake is obstructed by p-chloromercuibenzenesulfonic acid (PCMBS), a potent inhibitor of the plasma membrane sucrose transporter in plants (Giaquinta, 1976; Riesmeier et al., 1992). The observed biphasic kinetic patterns for sugar uptake by plant tissues may result from

combined carrier and diffusional mechanisms, different cell types in tissue with dissimilar uptake rates, or reflect metabolism of sugars within cells in addition to transport across the plasma membrane. In sink potato tuber tissue, biphasic kinetics of glucose and fructose uptake represents a linear component of sugar transport across the plasma membrane superimposed on a second saturable component representing starch synthesis within cells (Wright and Oparka, 1990).

Forney and Breen (1986) reported that uptake of sucrose by tissue disks from green-white strawberry fruit displays biphasic kinetics, with a saturable component predominant at low concentrations (< 25 mM) and a linear component at higher concentrations. Uptake of glucose was more rapid and its kinetics revealed only the first component, which saturates near 200 mM. Fructose uptake showed a pattern similar to that of sucrose. The influence of the osmolarity of the incubation solution on (i.e. cell turgor) the role and kinetics of sugar uptake by strawberry fruit tissues has not been investigated.

Sugar carriers and transporters are likely present in the plasma membrane of cells in sink tissues where sugar is unloaded into the apoplast or in cells in which leaked sugars are retrieved (Oparka, 1990; Riesmeier et al., 1994). Molecular approaches have led to the cloning of sucrose and hexose transporters from plants using yeast complimentation techniques discussed earlier in this literature review (Bush, 1993). A glucose transporter has been cloned from *Arabidopsis* by Sauer et al. (1990). Identification of the cellular location and the molecular structure of sugar transporters is now possible, and such

information will enable significant strides to be made in our understanding of sugar uptake mechanisms and their role in sink activity.

CHAPTER 3

EXPANSION AND OSMOTIC ADJUSTMENT OF STRAWBERRY FRUIT DURING WATER STRESS

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3.1 Abstract

Expansion of green-white and red fruit in watered (control) and water-stressed greenhouse-grown strawberry (*Fragaria* x *ananassa* Duch. cv. Brighton) plants was monitored with pressure transducers. Green-white fruit expansion in control plants was rapid, showing little diurnal variation, whereas in water-stressed plants it occurred only during dark periods and fruit shrinkage occurred during the day. Red fruit were mature and failed to show net expansion. The apoplastic water potential (ψ_{aw}), measured with *in situ* psychrometers, in control plants was always higher in leaves than in green-white fruit. In stressed plants, ψ_{aw} of leaves was higher than that of green-white fruit only in pre-dawn measurements, corresponding to the period when these fruit expanded. To determine their ability to osmotically adjust, fruit were removed from control and water stressed plants and hydrated in water for 12 h at 4°C or 20°C and then solute potential at full turgor (ψ_s^{100}) was measured. Hydration at 4°C reduced enlargement of water-stressed green-white fruit by one-third compared to that at 20°C. Water-stressed fruit hydrated at either temperature showed osmotic adjustment with a ψ_s^{100} that was 0.28 MPa lower than that of control fruit. Mature leaves of water-stressed plants showed a similar level of osmotic adjustment, whereas water stress did not have a significant effect on the ψ_s^{100} of red fruit. Using a rapid dehydration methodology, solute potential (ψ_s) of green-white fruit was measured and plotted against its relative water content (RWC = [fresh weight - dry weight/fully turgid weight - dry weight] x 100). Water-stressed green-white fruit had a lower ψ_s for a given RWC than control fruit further, confirming the occurrence of

osmotic adjustment in stressed fruit tissue. The lack of a linear relationship between turgor pressure and RWC prevented the calculation of cell elasticity or volumetric elastic modulus ($\varepsilon = \Delta\psi_p/\Delta RWC \times 100$). Osmotic adjustment resulted in about a 2.5-fold increase in the levels of both glucose and sucrose in stressed fruit, contributing to about 17% of the decrease in total ψ_s. Even though green-white fruit showed osmotic adjustment in water-stressed plants, it was not sufficient to maintain fruit expansion during the day.

3.2 Introduction

Expansion of fruit requires, among other factors, an adequate flow of water to the organ and sufficient turgor to drive cell enlargement. The water status of fruit or other plant organs may be characterized by their water potential (ψ_w), which is frequently considered the sum of turgor potential (ψ_p) and solute potential (ψ_s), when the gravitational component is ignored (i.e. $\psi_w = \psi_p + \psi_s$) (Nobel, 1983; Kramer and Boyer, 1995). Water moves along a gradient toward lower ψ_w, created between organs and tissues by differences in evaporational losses, solute accumulation, and water demand caused by cell expansion (Nonami and Boyer, 1993). Diurnal fluctuation of expansion has been reported in many fruit, including apple (Trump, 1984) and peach (Berger and Selles, 1993) and is related to the difference in ψ_w between the stem and fruit. Johnson et al. (1992) showed the establishment of a ψ_w gradient favoring solution flow into tomato

fruit coincides with a rapid increase in fruit diameter; inverting the gradient caused the fruit to shrink.

Positive cell turgor is required for plant growth (Meyer and Boyer, 1981; Kramer, 1988). Since ψ_p is equal to ψ_w minus ψ_s, the accumulation of solutes in cells and lowering of ψ_s increases ψ_p, allowing turgor dependant processes to continue at progressively lower ψ_w. Increased elasticity of cell walls is a passive mechanism which allows decreases in cell volume with dehydration, slowing the rate of loss of turgor with decreasing ψ_w. Osmotic adjustment, which is the maintenance of positive cell turgor through the active accumulation of solutes within cells, is often a strategy by which plants tolerate water or salt stress (Morgan, 1984). Osmotic and elastic adjustment together can maintain symplastic volume and positive cell turgor in plants diurnally and during soil drying (Saliendra and Meinzer, 1991). Jones et al. (1985) suggested that osmotic adjustment likely occurs in fruit tissues, but this has not been reported in the literature.

Water accounts for about 90% of the total weight of a ripe strawberry fruit (Darrow, 1966) and, therefore, it is not surprising that even mild drought stress can greatly reduce fruit yield (Renquist, 1982a; Dwyer et al., 1987). Osmotic adjustment has been reported in leaves of the cultivated strawberry (*Fragaria* x *ananassa* Duch.) (Renquist et al., 1982a; Save et al, 1993), as well as in its progenitor species, *F. chiloensis* Duch. (Archbold and Zhang, 1991) and *F. virginiana* Duch. (O'Neill, 1983). Archbold and Zhang (1991) suggested that the greater degree of osmotic adjustment shown in *F. chiloensis* than *F. virginiana* contributes to continued growth and survival of

F. chiloensis during drought. No information is available on whether strawberry fruit on water-stressed plants undergo osmotic adjustment as a means to continue expansion.

In order to distinguish between osmotic adjustment and passive changes in cell solute contents, such as by accumulation of solutes through dehydration, ψ_s should be measured at a particular ψ_w or water content. For convenience and purposes of comparison, the level of osmotic adjustment is usually determined by contrasting the ψ_s of excised tissues from water-stressed and watered plants after a hydration step to bring the tissue to full turgor (i.e. $\psi_w = 0$) (Jones and Turner, 1978; Turner, 1988).

The degree of osmotic adjustment that occurs in plants in response to a water deficit can be assessed by rehydration of control and water-stressed plants, followed by rapid dehydration and measurement of tissue ψ_s and relative water content (RWC = [fresh weight - dry weight/turgid weight - dry weight] x 100) (Turner and Jones, 1978; Morgan, 1984). A change in RWC of a plant tissue represents the change in cellular volume (Morgan, 1984). When the ψ_s of tissue is plotted against its RWC, a more negative ψ_s for a given RWC (i.e. cell volume) of stressed compared to unstressed plants is interpreted as osmotic adjustment in stressed tissues (Turner and Jones, 1978). Plotting the change in calculated turgor pressure (i.e. $\psi_p = \psi_{aw} - \psi_s$) versus RWC allows an estimate of cell elasticity or volumetric elastic modulus ($\varepsilon = \Delta\psi_p/\Delta RWC \times 100$).

The measurement of osmotic adjustment in growing plant tissues adds an additional complication. In expanding organs, such as growing fruit, cell walls yield under relatively low pressure so that cell volume may increase during the rise to full turgor in the hydration step. If at harvest, cells of water-stressed growing tissue have a

higher concentration of solutes than comparable non-stressed cells, they may take up more water osmotically during hydration and expand to a larger volume, thereby diluting the accumulated solutes, increasing ψ_s, and masking evidence of osmotic adjustment. Milad and Shackel (1992) found that expanding fruit from prune trees before irrigation showed a greater increase in both volume and ψ_s upon hydration as compared to fruit hydrated 24 h after irrigation. Turner and Jones (1978) reported that after a hydration period that would have lead to dilution of solutes in stressed tissue, the ψ_s was still lower in expanding leaves of water-stressed sunflower plants at a given RWC than that of control plants, indicating that osmotic adjustment occurred in stressed leaves. If cell expansion is metabolically dependant, lowering temperature of tissue during the hydration step might reduce dilution of ψ_s. Decreased temperatures during fruit hydration would also reduced respiration rates and utilization of solutes that contribute to ψ_s of the tissue.

Understanding the relationship between ψ_w and fruit expansion and the role of osmotic adjustment in growth maintenance could contribute to genetic or cultural manipulations to optimize fruit growth and yield. The objectives of this study were to determine whether fruit expansion in strawberry was related to the difference between leaf and fruit ψ_w, and if fruit undergo osmotic adjustment during water-stress.

3.3 Materials and methods

Plant material and stress imposition - Plants of the day-neutral strawberry (*Fragaria* x *ananassa* Duch.) 'Brighton', were grown during winter and early spring months in 3.8-liter plastic pots in 1 soil: 1 coarse sand: 1 peat (by volume) in a shaded greenhouse maintained at 30/18°C (day/night) in which high-pressure sodium lamps (HPS) provided supplemental light (175 ± 25 mmol m^{-2} s^{-1}) and a 14-h photoperiod, from 0730 to 2130 HR. The single primary flower on each inflorescence was pollinated with a small paint brush, tagged, and all competing flowers or buds at lower orders removed. Plants were watered daily and fertilized with 20N-8.6P-16.6K with N at 400mg $liter^{-1}$ twice weekly. At the start of each experiment, water was withheld from a portion of the plants to produce water-stress, whereas control plants continued to be watered daily.

Fruit expansion - Fruit expansion at green-white (15-20 days after anthesis (DAA)) and red (25-35 DAA) stages was monitored using linear variable differential transformers (LVDT) (model LD400-2.5, Omega Engineering, Stamford, Conn.) in conjunction with a micrologger (model 21X, Campbell Scientific, Logan, Utah). Each LVDT was secured in a custom-built U-shaped plexiglass platform which was placed over a single attached fruit positioned horizontally on a small stand. The plunger of the LVDT rested on the fruit from above, at about fruit midlength, and its weight was sufficient to maintain contact without damaging the fruit surface. An excitation voltage of 10vdc was maintained using an in-line voltage regulator. Expansion measurements were recorded diurnally every 10 min. Day measurements were corrected for a slight, but consistent, sensitivity of LVDTs to the HPS lamps.

Water relation components - Apoplastic water potential (ψ_{aw}), which is considered to represent ψ_w of a tissue (Nobel, 1983), was measured in fruit as described by Pomper and Breen (1995; see Chapter 4) using temperature-corrected *in situ* psychrometers (Dixon and Tyree, 1984) that were calibrated according to the manufacture's specifications (Plant Water Status Instruments, Guelph, Ontario, Canada) and used in conjunction with a microvoltmeter (model HR-33T, Wescor Inc. Logan, Utah). Psychrometers were attached with silicone grease to a site on a fruit where achenes had been removed with a forceps or to leaves where the cuticle was removed with fine sandpaper (600 grit), the surface was rinsed with distilled water and wiped dry. Plants had 10 to 15 leaves, of which one fully expanded, mature leaf was randomly chosen for ψ_{aw} measurement. A period of 3 h was found to be adequate for fruit tissue to reach equilibrium with the air in the psychrometer chamber (data not shown).

To monitor the diurnal fluctuation in ψ_{aw}, psychrometers were attached to a leaf and a green-white fruit on the same plant under water-stressed or control conditions at least 5 h prior to the first measurement taken in the evening (2100 HR); subsequent measurements were at predawn (0600 HR) and in the mid afternoon (1500 HR). Water was withheld from stressed plants for 5 to 7 days prior to attachment of psychrometers and they displayed a slight wilting during afternoon measurements. HPS lamps did not create an electrical interference with psychrometer measurements. Diurnal leaf and fruit ψ_{aw} measurements were replicated on four separate, cloudy days over a several week period. Leaf and fruit ψ_{aw} data were subjected to analysis of variance using a repeated measures design blocked by day. Leaf stomatal conductance and quantum flux were

measured with a steady state porometer (model LI-1600, LI-COR Inc., Lincoln, Neb.) using a broadleaf chamber (aperture set at 2 cm^2) as specified by the manufacturer.

Fruit solute potential (ψ_s) was determined after ψ_{aw} of fruit was measured at 1500 HR. Portions of fruit were place in liquid nitrogen for 15 min, then thawed for 20 min at room temperature, and cell sap obtained by centrifugation (3000x *g* for 5 min) at 4°C. After 3 min at room temperature, a portion of the cell sap was put on a 0.32 cm^2 filter paper disk (Wescor Inc.) which was inserted into a sample holder, provided by the manufacturer, and y_s determined psychometrically. The average cell ψ_p of a fruit was calculated (i.e. $\psi_p = \psi_{aw} - \psi_s$).

Osmotic adjustment - Two different methods were employed to determine whether stressed fruit underwent osmotic adjustment in plants subjected to water stress. First, to determine whether osmotic adjustment occurred in water-stressed fruit and leaves, control and slightly wilted stressed plants used in ψ_{aw} measurements were watered at 1500 HR and allowed to recover for 3 h in black plastic bags away from light (at which point leaves of stressed plants appeared fully turgid). Red and green-white fruit and fully expanded mature leaves (usually two per plant) were severed, still in plastic bags, and taken to the laboratory. The pedicel of red fruit and leaf petioles were re-cut under water and placed in beakers with distilled water. Water-saturated paper towels were placed around the beakers which were then covered with a black plastic bag to maintain a water saturated atmosphere. Red fruit were weighed at 0, 1, 3, 8, 10, and 12 h after the start of hydration. After 12 h, a leaflet or portion of a fruit was put in a microfuge tube (1.5 ml) which was then placed in liquid nitrogen for 30 min, thawed for 20 min at room

temperature, cell sap obtained by centrifugation, and solute potential at full turgor (ψ_s^{100}) determined psychometrically (Jones and Turner, 1978). The ψ_s^{100} of mature leaves (15 leaves per treatment) and red fruit (four fruit per treatment) was determined on three separate occasions and subjected to an analysis of variance in a randomized block design, blocked by day.

In a preliminary experiment, green-white fruit were found to take up appreciable amounts of water (gain fresh weight) upon hydration, raising concerns about a possible differential increase in cell volume between stressed and control fruit. From fruit weight and water displacement data (not shown), fruit volume in 'Brighton' was found to increase linearly with increasing fresh weight ($y = -0.264 + 1.225x$, $r^2 = 0.98$) from small green to red stages.

The effectiveness of low temperature in reducing fruit expansion during hydration was examined. Hydration at low temperatures would also reduce respiration rates and utilization of solutes that contribute to tissue ψ_s. For hydration, green-white fruit were cut in half lengthwise with a razor blade and half of each pair placed in distilled water in a petri dish at 4°C or 20°C. Fruit halves were weighed prior to hydration and after 1, 3, 8, 10 and 12 h. At the end of 12 h, a portion of the fruit was frozen in liquid nitrogen and ψ_s^{100} determined psychometrically as described above. Data for volume change and ψ_s^{100} were subjected to analysis of variance using a split-plot design. Four half-fruit were used for water-stressed and hydration treatment combinations.

A second method, basically that described by Turner and Jones (1978) except that fruit ψ_{aw} was determined psychometrically, was also used to determine whether tissues

underwent osmotic adjustment. Briefly, water-stressed and control plants with green-white fruit were watered in early evening, taken to the laboratory, and allowed to recover for 6 h, by which time wilted leaves of stressed plants became turgid. Psychrometers were attached to fruit as described above and measurements started after a 3 h recovery period. After an additional 3 h fruit, which were securely clamped to psychrometers, were severed from plants. The ψ_{aw} of detached fruit was measured every 6 h, after which one previously water-stressed and control fruit was sacrificed for ψ_s and RWC measurements. ψ_s was determined psychometrically with one portion of a fruit, a second portion (II) was weighed and then dried, and a third portion (III) placed in distilled H_2O for 3 h at 4°C and then weighed and dried. RWC was calculated as = $[(FW_{II} - DW_{II})/(TW_{III} - DW_{III})] \times 100$, where FW_{II} was initial fresh weight of the second portion, TW_{III} turgid weight of portion III after 3 h hydration, and DW the dry weight of oven dried portions of the fruit. Volumetric elastic modulus ($\varepsilon = \Delta\psi_p/\Delta RWC \times 100$) was determined by plotting the calculated turgor pressure for each green-white fruit from water-stressed and watered plants versus its RWC.

Sugar analysis - Hexoses and amino acids often are accumulated in plant tissues that osmotically adjust (Meyer and Boyer, 1981; Zhang and Archbold, 1993; Wang et al., 1995). Glucose and sucrose content was measured in control and water-stressed green-white strawberry fruit. In an effort to avoid cell damage that could induce hydrolysis of sucrose, fruit were not sectioned and re-hydrated in distilled water as previously described. Green-white fruit were harvested 6 h after water-stressed and control plants were re-watered and placed in black plastic bags. One portion of fruit was used to

determine ψ_s as described above and another portion was placed in boiling 70% ethanol for 5 min. The ethanol extract was taken to dryness, reconstituted in distilled H_2O, and glucose and sucrose levels determined as described by Pomper and Breen (1995; Chapter 4).

3.4 Results

Fruit expansion - In well watered plants, green-white fruit continually expanded, increasing at a rate of approximately 2.4 mm d^{-1} over the 3 days of the experiment, whereas control red fruit expanded slightly at first, but showed shrinkage by the last day (Fig. 3-1). As water-stress progressed it greatly diminished the growth of green-white fruit, and they showed increasingly conspicuous diurnal fluctuations in expansion. Although the nighttime expansion rate of green-white fruit was similar for water-stressed and control fruit, growth of stressed fruit ceased during daylight hours and shrinkage was often recorded. Shrinkage in green-white fruit was especially marked on the third day of measurement when stressed plants were first observed to wilt. Red fruit on water-stressed plants failed to show net expansion, but did exhibit a slight diurnal pattern of expansion and contraction.

Water relations - In another study, the expansion of two green-white fruit each on control and water-stressed plants was monitored over a 24 h period starting 4 days after withholding water from stressed plants. As before, fruit on well watered control plants showed rapid growth both during day and night, whereas expansion of water-stressed

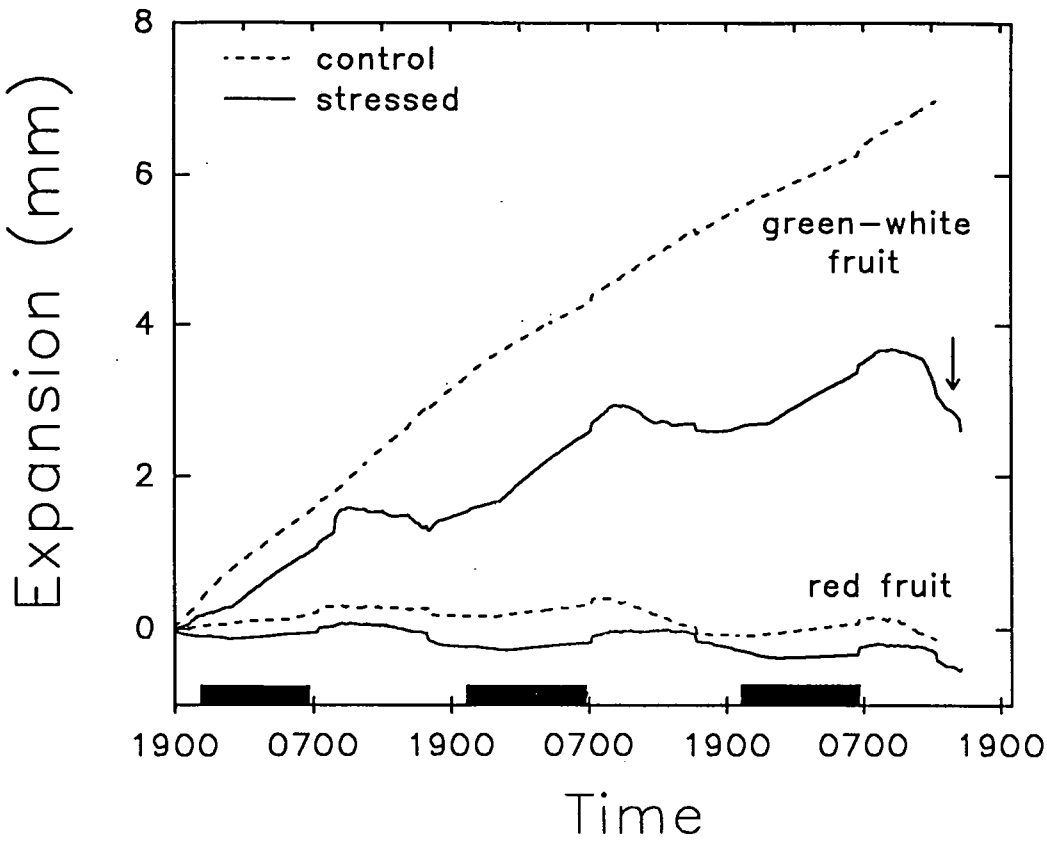

Figure 3-1. Expansion of green-white and red 'Brighton' fruit in well watered control (-----) and water-stressed (——) plants recorded with pressure transducers in the greenhouse. Water was withheld for 4 days prior the start of monitoring expansion. Data is from one representative experiment. Bars on x-axis indicate dark periods. Arrow indicates the beginning of wilting of leaves on stressed plant.

fruit occurred only at night and shrinkage was generally observed during daylight hours (Fig. 3-2A).

Fruit and leaf ψ_{aw} of these and two similar plants were determined over the same 24 h period. Leaf temperatures and light levels (quantum flux) were fairly consistent day to day during measurement periods in this study (Table 3-1). Their values, as well those of stomatal conductance, were highest at the mid-afternoon (1500 HR) sampling time.

As expected, stomatal conductance during daylight was much lower in stressed than control plants. Diurnal fluctuation in ψ_{aw} was greater in leaves than fruit (Fig. 3-2B). The ψ_{aw} of water-stressed leaves was significantly more negative than leaves of control plants. The ψ_{aw} of both control and water-stressed leaves significantly increased at 0600 HR and declined by 1500 HR. There was not a statistically significant interaction between treatment and time of day (see Appendix F). Water stress also lowered fruit ψ_{aw}, but the effect was not significant. Fruit ψ_{aw} did decline significantly from 0600 HR to 1500 HR in both control and stressed plants. Water-stressed plants showed wilting by 1500 HR, and green-white expanded little during light periods, indeed shrinkage was observed.

Water flow to, and expansion of, strawberry fruit may be associated with the differences in ψ_{aw} between leaves and fruit, therefore, these differences in control and water-stressed plants at the three sampling times are presented in Figure 3-3. The difference in ψ_{aw} between leaves and fruit was influenced significantly by water-stress and the time of day. In watered control plants the differences were always positive (i.e., ψ_{aw} of the leaf was higher, less negative, than that of fruit), with the smallest difference occurring in mid-afternoon. This positive difference corresponded with the relatively rapid expansion of green-white fruit on control plants (Fig. 3-2A).

In stressed plants, the difference in ψ_{aw} between the leaf and fruit was negative (i.e., ψ_{aw} of the leaf was less than that of fruit) at the two daylight measurements (2100 HR and 1500 HR), representing periods when fruit shrinkage predominated. However, the difference was positive at predawn (0600 HR), when expansion of fruit on stressed

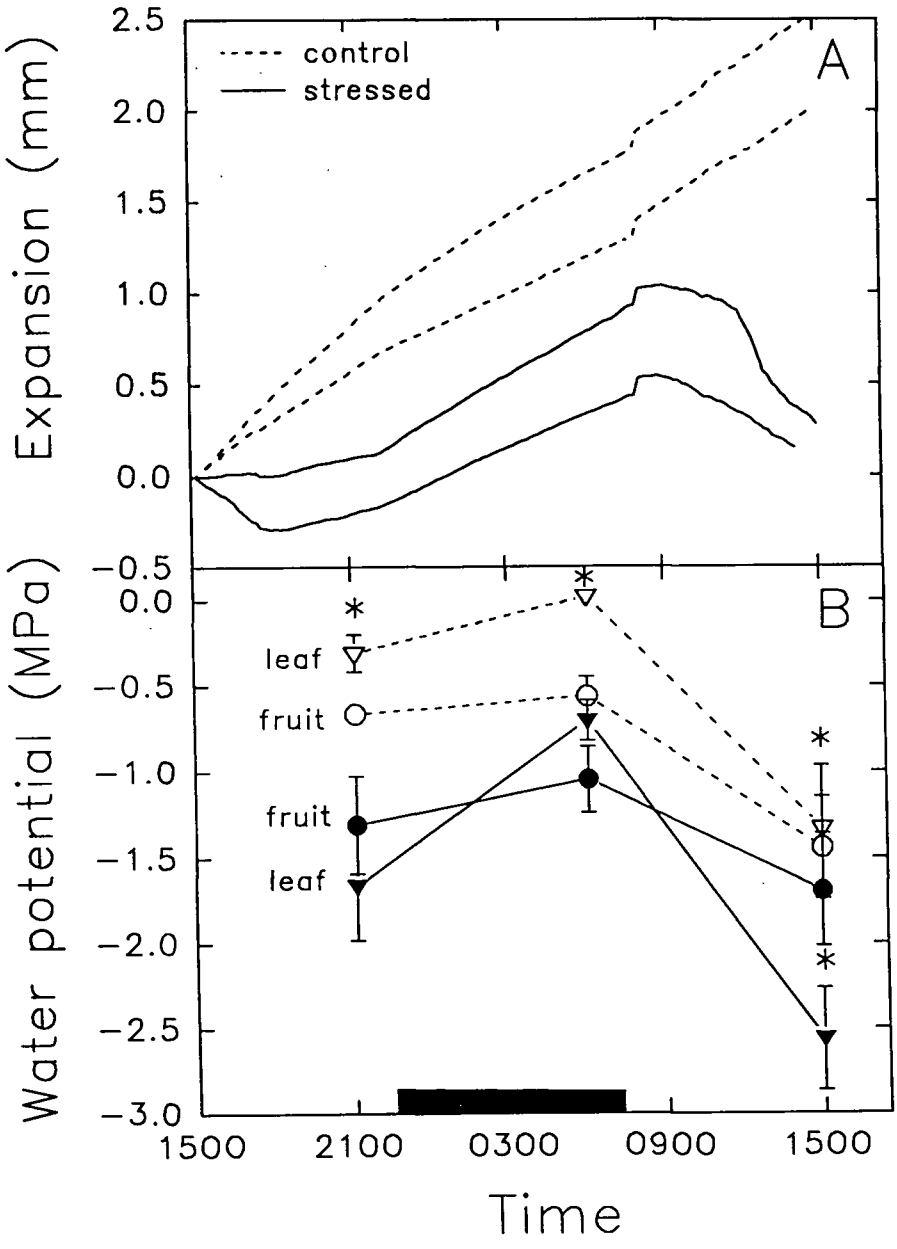

Figure 3-2. (A) Expansion of green-white 'Brighton' fruit in watered (-----) and water-stressed(——) plants recorded with pressure transducers in the greenhouse on two separate occasions. (B) The apoplastic water potential (ψ_{aw}) of leaves and green-white fruit in watered and water-stressed plants. Stars indicate means are significantly different at P=0.05 according to Student -Neuman-Keuls test. ±SE of four separate daily means.

Table 3-1 Leaf temperature, stomatal conductance, and quantum flux during fruit and leaf water potential (ψ_{aw}) and fruit expansion experiments.

Time (HR)	Leaf temperature (C°)	Quantum flux (mmol $m^{-2}s^{-1}$)	Stomatal conductance (cm sec^{-1})	
			Control	Water-stress
2100	21.1 ± 0.3*	137.0 ± 10.5	0.16 ± 0.02	0.02 ± 0.01
0600	16.2 ± 0.3	0.0 ± 0.0	0.00 ± 0.00	0.00 ± 0.00
1500	24.1 ± 0.8	228.0 ± 58.3	0.53 ± 0.05	0.13 ± 0.02

* Note: mean derived from four separate experiments. ±SE

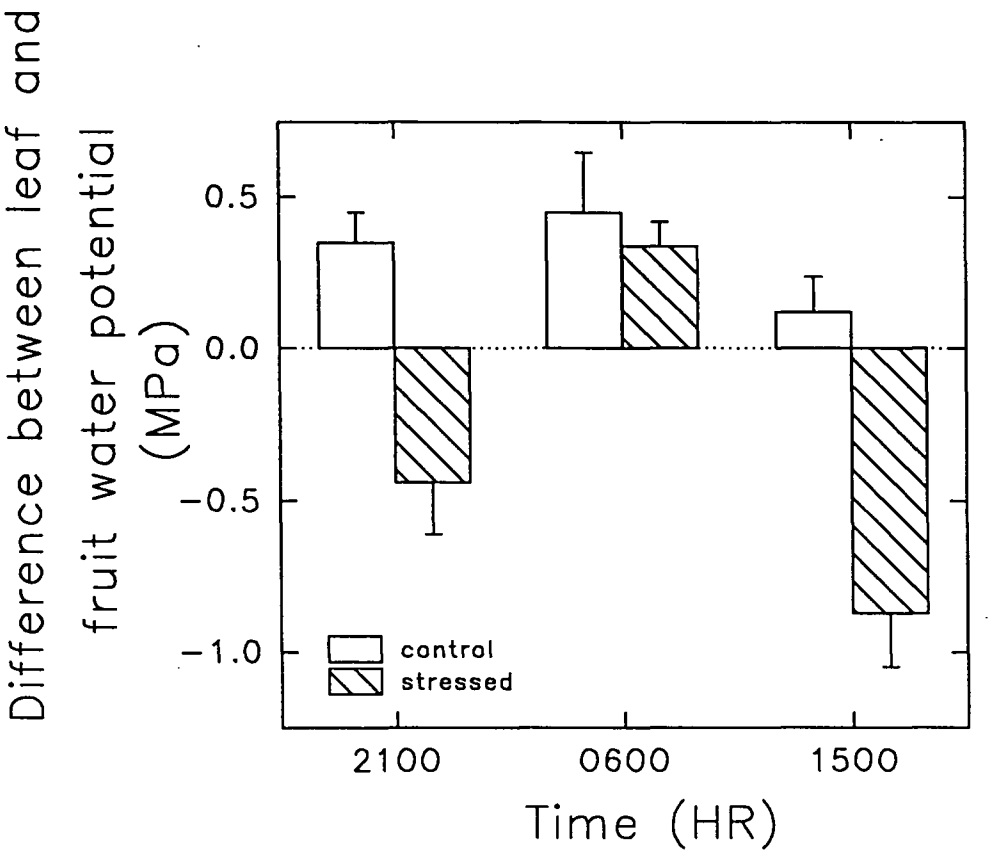

Figure 3-3. The difference between leaf and fruit apoplastic water potential (ψ_{aw}) (e.g. $\Delta \psi_{aw} = \psi_{leaf} - \psi_{fruit}$) for watered (□) and water-stressed (▨) 'Brighton' strawberry plants. ±SE of four separate experiments.

plants was rapid. Fruit were harvested from plants after the ψ_{aw} measurement at 1500 HR and ψ_s determined, allowing ψ_p to be calculated ($\psi_p = \psi_{aw} - \psi_s$). Green-white fruit from control plants in mid-afternoon had a positive ψ_p of 0.33 MPa, which was significantly higher than that of fruit from stressed plants in which the calculated ψ_p was negative, -0.88 MPa (mean separation by Student-Neuman-Keuls, $P < 5\%$).

Osmotic adjustment - Fruit halves from both control and stressed green-white fruit increased in volume during hydration at either 20°C and 4°C, reaching maximum volume by 10 h (data not shown). The volume increase in control fruit was significantly less at the lower hydration temperature, with mean values of 0.14 and 0.09 ml ml^{-1} for 20°C and 4°C, respectively (mean separation by Student-Neuman-Keuls, $P < 5\%$). Similarly, the volume of stressed fruit upon hydration increased to 0.26 ml ml^{-1} at 20°C, but only 0.18 ml ml^{-1} at 4°C. Since the increase in fruit volume was about 30% less at a hydration temperature of 4°C than 20°C, strawberry fruit expansion during hydration may be partially metabolically dependant. Both treatments (control or water-stress) and hydration temperature had significant effects on volume change of green-white fruit upon hydration, however, they did not show a significant interaction (see Appendix F).

Osmotic adjustment occurred in mature leaves of stressed plants, shown by a significantly lower ψ_s^{100} (0.27 MPa) than control leaves (Table 3-2). Water-stress did not cause significant osmotic adjustment in red fruit, although ψ_s^{100} was lower in stressed than control fruit (Table 3-2). The volume increased in red fruit during hydration was slight (0.001 ml ml^{-1}) in both treatments. Hydration temperature (20°C or 4°C) did not significantly effect the ψ_s^{100} of green-white fruit, so the data were combined for statistical analysis (see Appendix F). Green-white fruit from water-stressed plants showed evidence of osmotic adjustment, demonstrated by a significantly lower ψ_s^{100} (0.28 MPa) than fruit from control plants (Table 3-2).

Table 3-2. Solute potential at full turgor (ψ_s^{100}) of leaves and red fruit, hydrated at 20°C and green-white fruit hydrated at 4° and 20°C, from 'Brighton' strawberry under water-stressed or watered (control) conditions.

Treatment	Solute potential at full turgor (ψ_s^{100})		
	Leaf	Red fruit	Green-white fruit
		MPa	
Control	-1.30 a[2]	-0.94 a	-1.00 a
Water-stressed	-1.57 b	-1.07 a	-0.72 b
Difference	0.27	0.13	0.28

[1]After hydration of tissue from control or stressed plants, portions of the material were frozen in liquid nitrogen, thawed and ψ_s^{100} measured from the cell sap via psychrometry.

[2]Separation of means by Student-Neuman-Keuls separation of means at a level of significance of 5%, n = 3.

The ψ_s did not decrease with a reduction in RWC with green-white fruit of control or water-stressed plants using the method of Turner and Jones (1978) (Fig. 3-4A). However, ψ_s of water-stressed green-white fruit was usually below -1.4 MPa and more negative than that of control fruit at a given RWC, indicating that osmotic adjustment occurred in water-stressed fruit. The lack of a linear relationship between turgor pressure and RWC prevented the calculation of cell elasticity or ε (Fig. 3-4B). Calculated ψ_p was negative for most control and water-stressed fruit after dehydration.

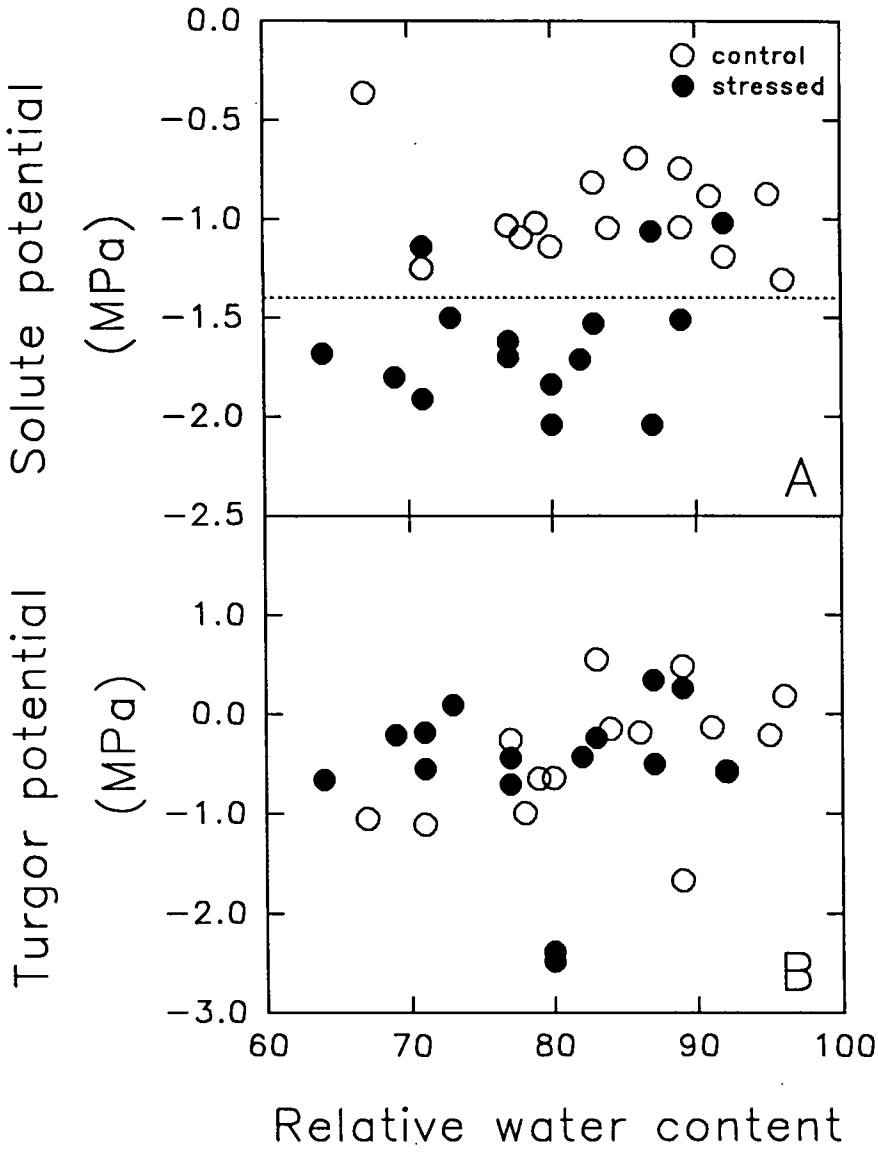

Figure 3-4. (A) The relationship between solute potential (ψ_s) and relative water content (RWC) during rapid drying for green-white fruit from watered (O) and water-stressed (●) plants. (B) The relationship between turgor potential (ψ_p) and RWC during rapid drying for green-white fruit from watered (O) and water-stressed (●) plants. The dashed line emphasizes that a majority of fruit from water-stressed plants display ψ_s below -1.4 MPa.

Sugar analysis - The ψ_s of green-white fruit from water-stressed plants was -1.41 MPa, which was significantly more negative than that of control plants at -0.82 MPa (P-value< 0.05 mean separation using independent t-test; n = 5). Both glucose and sucrose levels were several fold higher in stressed than control green-white fruit. Control fruit had levels of glucose at 2.7 ±0.2 mmol gFW^{-1} and sucrose at 4.3 ±0.4 mmol gFW^{-1}, whereas water-stressed fruit had glucose at 12.7 ±2.7 mmol gFW^{-1} and sucrose at 12.1 ±1.3 mmol gFW^{-1} (each individual sugar was significantly higher in stressed fruit with a P-value< 0.05 mean separation using independent t-test; n = 5). The ratio of glucose to sucrose was 1:1.5 in control and 1:1 in stressed fruit, indicating that some hydrolysis of stored sucrose may have occurred. Sugar concentrations in green-white fruit wer calculated by assuming that the water content of fruit was 90% (based on fruit growth data in Appendix D in Fig. D-1) at full turgor. The concentration of glucose was 3.0 mM (e.g. 2.7×10^{-3} mmol / 9.0×10^{-4} liter) and that of sucrose 4.6 mM in fruit of control plants, whereas values were several fold higher in fruit from water-stressed plants at 13.9 mM and 13.4 mM for glucose and sucrose respectively.

3.5 Discussion

Strawberry fruit expansion was very sensitive to changes in plant water status. Even 3 days prior to leaf wilting, fruit expansion on water-stressed plants was inhibited in daylight conditions. Diurnal expansion of green-white fruit on these plants likely reflected increased water demand by leaves (even with reduced opening of stomata) and limited soil moisture. In a laboratory study, Antoszewski (1974) reported that

illumination of strawberry leaves caused contraction in green-white and red fruit, coinciding with opening of leaf stomata; the water status of plants used was not defined. In the present study, red fruit were not expanding and showed little diurnal expansion or contraction (Fig. 3-1).

The observed shrinkage of green-white fruit may reflect movement of water back into the plant crown or limited water transport to the fruit with continued water loss from its surface. Fruit shrinkage has been reported in other species and has been taken to represent back flow of water into the stem (Trump, 1984; Johnson, 1992; Berger and Selles, 1993). Water flux from apple fruit toward the trunk during plant water stress has been measured using a heat-pulse method (Yamamoto, 1983). Grange and Andrews (1995) reported that tomato fruit show diurnal fluctuation in expansion, growing during the night and shrinking during the day, even in well watered plants.

Strawberry fruit expansion occurred when leaf ψ_{aw} was higher than that of fruit, supporting the idea that water flows to regions of lower ψ_{aw} in the plant. The greater diurnal variation in ψ_{aw} in strawberry leaves than fruit reported here (Fig. 3-3) is similar to what Johnson (1993) described for tomato, where fruit expansion and shrinkage was correlated with diurnal fluctuations in stem ψ_{aw}. Interestingly, strawberry fruit ψ_{aw} was significantly higher than that of leaves in stressed plants in the afternoon, suggesting that fruit were somehow buffered or isolated from extreme changes in ψ_{aw} of the xylem stream.

Water is expected to flow to regions of lower ψ_{aw} in a plant, but this assumes an unrestricted path of water movement. There is evidence that this is not always the case,

and therefore, that ψ_{aw} may not a good predictor of water transport in plants (Passioura, 1988). Clearly there are mechanisms that either isolate organs from the xylem stream or somehow provide preferential water transport to certain regions. Trolinder et al. (1993) reported that petals of cotton flowers continue to expand when leaf expansion is inhibited by water stress, with ψ_w of petal tissue being consistently higher that of subtending leaves and bracts. In some manner, water needed for petal expansion is independent of the ψ_w gradient within the cotton plant. In wheat and barley, grain ψ_w remains about 1 MPa higher than that of associated bracts during water stress (Barlow et al., 1980; Brooks et al., 1982). Barlow et al. (1980) reported that the deposition of lipids and suberin in the chalazal end of the wheat kernel was enhanced by water stress and may serve to isolate the grain hydraulically from the mother plant, preventing a fall in ψ_w in this organ under water stress. They also suggested that the phloem may be the major path of delivery of water to the isolated grain.

Phloem appears to be an important vascular component for water delivery into fleshy fruits (Findlay et al., 1987; Lee, 1990b). The ability of strawberry fruit to maintain a steady ψ_{aw} under water stress could be related to the development of a hydraulic barrier to the xylem stream or some other isolation mechanism. Pomper and Breen (1995) found evidence of an apoplastic barrier to solute movement in strawberry fruit when, using a pressure chamber, they were unable to retrieve a xylem mobile dye from the pedicel after it was injected into the body of the fruit. Antoszewski (1974) reported that cavitation greatly increases in the strawberry fruit pedicle upon illumination of leaves, such a process could effectively isolate the fruit from the xylem stream of the rest of the plant.

It was not unexpected that turgor was low in green-white from water-stressed plants. However, the negative turgor value (-0.88 MPa) calculated for such fruit in mid-afternoon, when turgor of control fruit was positive, probably resulted from an overestimation of fruit ψ_s and or an underestimation of ψ_{aw}. The calculated turgor of fruit subjected to rapid drying was also negative (Fig. 3-4B). In tissues with low turgor, such as those under water stress, negative ψ_p values may result (Wenkert, 1980). The freeze-thaw step used to disrupt cells prior to measuring ψ_s permits dilution of cell solutes with apoplastic water raising ψ_s, making it less negative, and lowering calculated ψ_p values. Since we were unable to estimate the extent of dilution of cell solutes by solution in the apoplast in strawberry fruit, we did not attempt to mathematically adjust ψ_s values. Additionally, there may be ψ_w gradients within severely water-stressed strawberry fruit, and the ψ_{aw} of the outer region of the fruit measured with *in situ* psychrometers may have been more negative than internal values, thereby lowering calculated turgor values of stressed fruit. Milad and Shackel (1992) found differences in ψ_s of cell sap measured between the stem and stylar ends of prune fruit. Differences in ψ_s of cells within the strawberry fruit could also cause negative calculated turgor values for whole fruit. Whatever the reason for the negative turgor calculation for green-white strawberry fruit on water-stressed plants, these values indicate that fruit cells were likely at very low turgor.

Increases in cell volume during growth need to be carefully distinguished from reversible elastic effects of cell walls, so plant growth is defined as an irreversible increase in size to separate it from the reversible changes (Kramer and Boyer, 1995). In

mature tissues, elastic changes are the only ones that occur in cell walls and are easily detected; they are reported in terms of ε (Kramer and Boyer, 1995). Cell growth is often analyzed according to the equation formulated by Lockhart (1965): $r = m (\psi_p - Y)$, where r is the rate of increase in cell volume, m is the extensibility of the cell wall (irreversible extension), ψ_p is the turgor, and Y is the minimum turgor below which no expansion occurs (see Passioura, 1994a). This equation is most useful for short-term responses of several minutes, with m and Y adjusting to changes in ψ_p. The effects of increased turgor on cell expansion are transient, with continued expansion dependant on new cell wall synthesis (Shackel, 1987; Passioura, 1994b). Cell expansion is likely dependant on cell wall enzymes that cleave load-bearing hemi-cellulose molecules, reducing wall stress, and allowing growth (Cosgrove, 1993; Passioura, 1994b). If turgor falls to zero, no cell expansion will occur.

Osmotic and elastic adjustment together may be important in maintaining symplastic volume and positive cell turgor in mature tissues of droughted plants (Bressan, 1990; Saliendra and Meinzer, 1991). However, the elasticity of cell walls in tissues that osmotically adjust with drought often show decreases in elasticity, such as in mature strawberry leaves (Save et al., 1993), while in other plants, tissue elasticity increases (Saliendra and Meinzer, 1991) or stays the same (Jones and Turner, 1980). Elasticity of growing tissue is difficult to measure, since even small increases in the cell ψ_p cause extension of the cell walls. Several groups have reported that changes in elasticity of cell walls in young expanding leaves that have undergone osmotic adjustment (Jones and Turner, 1980; Grima and Krieg, 1992). Since there was no clear relationship between

turgor pressure and RWC in green-white strawberry fruit (Fig. 3-4A), we were unable to determine whether there were differences in ε of watered and water-stressed green-white fruit. Growth of green-white fruit during hydration, even at low temperature, likely caused unreliable measurements of RWC.

This laboratory continues to be interested in the uptake of glucose and sucrose by fruit tissues (Pomper and Breen, 1995; Pomper and Breen, 1996). Sucrose is the major sugar translocated in strawberry (Forney and Breen, 1985) and glucose, fructose, and sucrose accumulate to about equal levels (1% FW) in green-white 'Brighton' strawberry fruit (Forney and Breen, 1986). Using the van't Hoff equation (e.g. ψ_s = -Conc. RT at 25°C) and a total concentration of glucose and sucrose of 7.0 mM in control fruit and 25 mM in water-stressed fruit, the total ψ_s of glucose and sucrose in the fruit can account for about -0.018 MPa and -0.067 MPa in control and water-stressed fruit, respectively. Only about 2% of the ψ_s of control fruit (-0.82 MPa) and about 5% of the total ψ_s of water-stressed fruit (-1.41 MPa) was accounted for by glucose and sucrose in the fruit. Osmotic adjustment resulted in about 2.5-fold increase in the levels of these sugars in stressed green-white fruit but this contributed only about 17% of the decrease in ψ_s in stressed fruit. Intracellular invertase activity, which hydrolyses sucrose into glucose and fructose, is present in green-white fruit of 'Brighton' (data not shown) and developing fruit of other cultivars (Hubbard, et al., 1991). The increase in glucose compared to sucrose in water-stressed green-white strawberry fruit may have resulted from the hydrolysis of stored sucrose or reduced sucrose transport.

In conclusion, strawberry fruit expansion was very sensitive to water stress and occurred when leaf ψ_{aw} was higher than that of fruit. Although, green-white fruit underwent osmotic adjustment when plants were subjected to water-stress, this was not sufficient to maintain fruit expansion during daylight hours when stress was greatest. Growth of green-white fruit during hydration, even at low temperature, caused unreliable measurements of RWC which did not allow calculation of ε. Osmotic adjustment resulted in a 2.5-fold increase in the levels glucose and sucrose in stressed green-white fruit, but this contributed only about 17% of the decrease in ψ_s in stressed fruit.

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CHAPTER 4

LEVELS OF APOPLASTIC SOLUTES IN DEVELOPING STRAWBERRY FRUIT

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4.1 Abstract

Solutes in the apoplast of sink organs can contribute to apoplastic water potential (ψ_{aw}) gradients that influence transport of water and assimilates. However, sampling the apoplast solution is often technically difficult. Three methods were used to collect apoplastic solution from developing strawberry fruit for determining apoplastic solute potential (ψ_{as}) and sucrose and glucose concentrations. In addition, ψ_{as} was estimated from the difference between ψ_{aw} (measured via hygrometry) and fruit xylem pressure potential ($\psi_{x\text{-}press}$) (measured with a pressure chamber). As strawberry fruit developed, ψ_{aw} decreased linearly whereas $\psi_{x\text{-}press}$ declined slightly then increased, therefore the estimated ψ_{as} also decreased. Liquid was recovered from tissue plugs of ripening fruit using centrifugation. The solute potential (ψ_s) of these samples from pink and red fruit varied from -0.9 to -1.1 MPa and was similar to that of the bulk fruit solute potential ($\psi_{s\text{-}bulk}$). A novel method was developed to sample the apoplastic solution, with little or no cell damage, by collecting liquid on filter paper disks inserted into the hollow cavity naturally formed in ripening fruit. Here the ψ_s was about -1.0 MPa, again similar to $\psi_{s\text{-}bulk}$, and sucrose and glucose concentrations were each near 50 mM. Solution recovered from ripening fruit by centrifugation and paper disks from the fruit cavity was red, suggesting leakage of anthocyanins into the apoplast. Liquid collected from the pedicel xylem of detached fruit using a pressure chamber was colorless, had a high ψ_s (about -0.3 MPa), and low sugar concentrations (< 1 mM) throughout fruit development,

suggesting that this method is not effective for sampling the fruit apoplast. Sugars accounted for only about 35% of the ψ_s of the apoplast of ripening strawberry fruit.

4.2 Introduction

Solutes are present at high levels in the apoplast (the whole continuum outside the plasma membrane) of sink tissues such as mature sugarcane stalks (Welbaum and Meinzer, 1990), developing seeds (Wolswinkle, 1990), and ripening grape berries (Lang and During, 1991). The apoplast can serve as a storage area for sugar; twenty percent of the sucrose in mature sugarcane tissue is held in the apoplast, reaching concentrations of 700 mM (Welbaum and Meinzer, 1990). High concentrations of solutes in the apoplast may create apoplastic water potential (ψ_{aw}) gradients within plants, influencing water and assimilate flow from source to sink organs.

Sugar and other solutes are translocated in the phloem by a passive bulk flow down a gradient of hydrostatic pressure from source to sink regions. Lang and Thorpe (1986) have hypothesized that high levels of solutes in the apoplast could promote removal of water from the phloem, reducing its turgor, and increasing the efflux of translocated solutes into the apoplast for subsequent uptake by sink cells. Apoplastic water would then move into cells due to their increased solute content, allowing continued water and assimilate import to the region. These authors found that steepening the ψ_{aw} gradient between the shoot and root of *Phaseolus vulgaris* L., by bathing the root in a solution containing a non-permeating osmoticum, increased assimilate flow to the

root. A ψ_{aw} gradient between the stem and grape berry has been identified that is created by high levels of apoplastic solutes in developing berries, this at a period when reduced xylem conductivity prevents backflow of solutes in the fruit apoplast to the stem (Lang and During, 1992). High levels of solutes could be maintained in the fruit apoplast due to low rates of fruit transpiration, such as in tomato (Johnson et al., 1992), reducing xylem flow to fruit. The long diffusional path of the fruit pedicel would essentially isolate apoplastic solutes from the dilute xylem solution in the stem.

Solutes may leak from cells into the apoplast in sink tissue as they mature and senesce, as postulated by Lang and During (1991) for ripening grape berries. Ripening fruit of strawberry (*Fragaria* x *ananassa* Duch.) display extreme swelling of cell walls and degeneration of plastids (Knee et al., 1977), suggesting that a loss in cell membrane integrity with maturation could lead to an increase in apoplastic solute levels. It is possible to estimate, indirectly, the level of apoplastic solutes by assuming that the apoplastic solute potential (ψ_{as}) equals the difference between ψ_{aw} and $\psi_{x\text{-}press}$ (xylem pressure potential or tension) (i.e., $\psi_{as} = \psi_{aw} - \psi_{x\text{-}press}$). Preliminary work in our laboratory shows that the calculated values of ψ_{as} become more negative as strawberry fruit ripen, indicating increased accumulation of solutes in the apoplast. Direct, unambiguous quantification of solutes in the apoplast of plant tissues is technically difficult. This is especially so with fragile tissues such as those from strawberry fruit which are easily damaged. Washing-out (Glasziou and Gayler, 1972) or centrifugation of tissue segments (Terry and Bonner, 1980) have been used to obtain samples of the apoplast. However, a serious problem with these techniques is unforeseen cell damage

and release of cell constituents which contaminate the apoplast solution (Ehwald et al., 1980).

Collection of xylem exudate using a pressure chamber has also been used to sample the apoplastic solution (Jachetta et al., 1986). This approach may not always be suitable, for xylem discontinuity, such as occurs in grape (Findlay et al, 1987) and tomato (Lee, 1989) fruits, may restrict the movement of solutes between the apoplast and vascular system of the stem. However, Lang and During (1991) were able to collect xylem exudate that appeared to represent the fruit apoplast from ripening 'Riesling' grapes. Physical semi-permeable barriers surrounding vascular bundles may also interfere with the free movement of apoplastic solutes in storage organs, such as in sugarcane stalks (Welbaum, et al., 1992) and leaves of many plants (Canny, 1993), thereby preventing sampling of the apoplast via the pressure chamber technique.

Sucrose is the major sugar translocated in strawberry (Forney and Breen, 1985a) and may be apoplastically unloaded in the fruit (Forney and Breen, 1986), thereby contributing to solute levels in the apoplast. The subsequent uptake of sucrose from the apoplast by fruit cells could promote continued translocation to the fruit. Uptake of sucrose by strawberry fruit tissue *in vitro* displays biphasic kinetics, with a saturable (carrier mediated) component predominant at low sugar concentrations (< 25 mM) and a linear (diffusional) component at higher concentrations (Forney and Breen, 1986). Fructose uptake shows a similar pattern. However, uptake of glucose is more rapid and its kinetics reveal only the first component, which saturates near 200 mM. Determination of the *in vivo* composition and concentrations of sugars in the apoplast of strawberry fruit

would be helpful in ascertaining the contribution of saturable and linear uptake components in sugar uptake or retrieval by fruit cells.

Identification of mechanisms and pathways by which water and sugar are transported to strawberry fruit may allow manipulation of water and dry matter partitioning within the plant. The objectives of this study were to sample the apoplast of the developing strawberry fruit, to assess the contribution of solutes toward $\psi_{as,}$ and quantify apoplastic sucrose and glucose levels. Several techniques were used to sample the apoplastic solution of the strawberry fruit and a novel method was developed to obtain a sample of the solution with little or no cell damage.

4.3 Materials and methods

Plant material - Plants of 'Brighton', a day-neutral strawberry, were grown in 3.8 liter plastic pots in 1 soil: 1 coarse sand: 1 peat (by volume) in a greenhouse maintained at 30/18°C (day/night) in which high-pressure sodium lamps (175 ± 25 mmol m^{-2} s^{-1}) provided a 16-h photoperiod. The single primary flower on each inflorescence was pollinated with a small paint brush, tagged, and all competing flowers or flower buds at lower orders removed.

Apoplastic water potential - The ψ_{aw} was determined using temperature-corrected *in situ* hygrometers (Dixon and Tyree, 1984) that were calibrated according to the manufactures specifications (Plant Water Status Instruments, Guelph, Ontario, Canada) and used in conjunction with a microvoltmeter (model HR-33T, Wescor Inc. Logan, UT) as described

by Lee et al. (1988). Briefly, hygrometers were attached to the fruit surface where several achenes had been removed with a fine forceps, the fruit surface rinsed with distilled water, and wiped dry. Hygrometers were attached to green, green-white, pink, and red primary fruit, at 7, 15, 20, and 25 days after anthesis (DAA), respectively, using silicone grease at the instrument-fruit junction to ensure a vapor seal. The entire installation was covered with glass wool and pieces of styrofoam to minimize temperature shifts, and allowed to equilibrate overnight (10-12 h). Measurements of ψ_{aw} were made during the following afternoon in the greenhouse.

Fruit xylem water potential - The $\psi_{x\text{-press}}$ (or xylem tension) was determined for each fruit immediately after completion of ψ_{aw} measurements. The fruit was severed with a razor blade at the pedicel (with a 3 cm stub) and quickly placed in a pressure chamber (PMS Instruments, Corvallis, OR). A cut plastic pipet tip (200 ml) was temporarily inserted into one side of the rubber septum of the pressure chamber to minimize damage to the fruit pedicel inserted from the other side. Pressure was slowly increased (approximately 0.006 MPa s^{-1}; Brown and Tanner, 1981) until bubbles were evident from the pedicel xylem. The pressure at that moment was recorded as the fruit $\psi_{x\text{-press}}$. Preliminary studies showed that hygrometer attachment did not influence fruit $\psi_{x\text{-press}}$. Apoplastic solute potential was calculated for each fruit as $\psi_{as} = \psi_{aw} - \psi_{x\text{-press}}$.

Fruit solute potential - To determine bulk solute potential ($y_{s\text{-bulk}}$), portions of the same fruit used to determine ψ_{aw} and $\psi_{x\text{-press}}$ were frozen at -80°C for 1 h, thawed for 20 min at room temperature, cell sap obtained by centrifugation at 4°C (3000x *g* for 5 min), and sap osmolarity measured with a vapor pressure depression osmometer (Wescor 5100 C,

Wescor Inc.) (Li and Delrot, 1987). Throughout this work, osmolarity was converted to solute potential (25°C) by using 2.48 MPa per $Osmkg^{-1}$ (e.g. $\psi_{s\text{-}bulk}$ = -Conc.RT at 25°C = Osmol x 2.48). Cell turgor was calculated according to $\psi_{turgor} = \psi_{s\text{-}bulk} - \psi_{aw}$.

Xylem exudate collection - Xylem exudate was collected by a method developed for sunflower leaves (Jachetta et al., 1986). Fruit were excised during the afternoon, placed in a pressure chamber (with about 1 cm of pedicel protruding outside) and the cut pedicel end blotted dry. After an initial balance pressure was determined ($\psi_{x\text{-}press}$), pressure was increased in increments of 0.02 to 0.04 MPa. A period of 3 to 5 min was sufficient for collecting a suitable sample of sap, after which pressure was increased again to a new balance pressure to continue the efflux of xylem sap. The sap was absorbed on a filter paper disk (6 mm diameter) placed on the cut surface. It was determined that when a paper disk took on a distinctive glistening appearance it held approximately 7ml. To reduce evaporation from the paper disk, water saturated tissue paper was placed in the recessed area of the pressure chamber head, which was then covered with plastic wrap. Upon saturation, the disk was transferred immediately to the sample chamber of the vapor pressure osmometer and the measurement of osmolarity converted to the solute potential of xylem exudate ($\psi_{s\text{-}xylem}$) as described above. The total xylem water potential (ψ_{xwp}) was calculated as the sum of $\psi_{s\text{-}xylem}$ and $\psi_{x\text{-}press}$. Disks were then stored at -80°C for later glucose and sucrose analysis.

The terms $\psi_{x\text{-}press}$ or $\psi_{s\text{-}xylem}$ specifying the xylem pressure or solute potential are used as a convenience, since these measured parameters may also represent non-xylem apoplastic compartments within the fruit. When chamber pressure is increased to reach

or surpass the $\psi_{x\text{-}press}$ of the fruit (when fluid is pushed from the cut pedicel), water may be forced through cell membranes, diluting the solution in the fruit apoplast (see Passioura, 1980; Jachetta et al., 1986, Canny, 1993).

A dye was injected into strawberry fruit to test whether a xylem discontinuity or a physical barrier surrounding the xylem could interfere with the sampling of apoplastic solution. A xylem mobile dye, 1% (W/V) aqueous azosulfamide solution (Ashworth, 1982), was injected (20 or 200 ml) with a fine needled syringe into the cortex of three green fruit and the cortex and cavity of three green-white or red fruit. The fruit was then detached, placed in a pressure chamber, exudate collected as described previously, and the presence of dye noted.

Centrifugation of tissue - A solution from strawberry fruit tissue was collected by centrifugation using a method described by Welbaum and Meinzer (1990) with sugarcane stems. Fruit was harvested in mid-afternoon and immediately cut with a 1 cm-diameter cork borer and a 1 cm-long cylinder from the cortex placed in a microfilter centrifuge tube (1.2 cm diameter) equipped with a 0.8 mm cellulose acetate filter and a 3 ml receiver tube (Fisher Scientific, Pittsburgh, Penn.). Tubes were centrifuged for 10 min each at 30, 160, 300, 500, 750, 1000, 1200, 1600, 2800, and 4000x *g* (the limit of the microfilter centrifuge tubes), unless otherwise noted. Solution collected in the receiver tube was completely removed after each centrifugation, the osmolarity measured with the vapor pressure depression osmometer, and converted to the solute potential of the solution collected ($\psi_{s\text{-}cent}$). At the conclusion of the centrifugation series, the tissue cylinder was frozen at -80°C for 1 h, thawed for 20 min at room temperature, centrifuged at 4°C

(3000x *g* for 5 min), osmolarity obtained with the vapor pressure depression osmometer, and solute potential of the cell sap ($\psi_{s\text{-}bulk}$) calculated.

Collection from the fruit cavity - The anatomy of the strawberry fruit provides an opportunity to directly retrieve a sample of apoplastic solution from the fruit while attached to the plant. Vascular traces of the strawberry fruit radiate out to each achene from a central vascular cylinder surrounding the pith, which in some cultivars, including 'Brighton', develops a hollow cavity as the fruit enlarges. By carefully cutting the attached fruit along its longitudinal axis and partially separating the halves, with the fruit hinged near the calyx, it is possible to insert a filter paper disk (6 mm diameter) into the cavity in contact with undamaged pith cells to collect an un-contaminated sample of the apoplastic solution by capillary action (Fig. 4-1). This technique was used successfully to collect solution from the hollow cavity of use of this method. Filter paper disks were also used to collect solution from the cut surface of the fruit cortex. Four disks were collected from both the cavity and cut surface of each fruit. Filter paper disks placed in the fruit cavity were removed when they appeared to saturate (usually 15 to 30 minutes). The vapor pressure depression osmometer was used to determine solute potential for the fruit cavity ($\psi_{s\text{-}cavity}$) and surface ($\psi_{s\text{-}surface}$). The disks were then stored at -80°C for later glucose and sucrose analysis.

Glucose and sucrose analysis - Sugars were eluted from the filter paper disks into 500 ml of distilled water. One aliquot was assayed for glucose using the glucose oxidase, peroxidase and o-dianisidine system from Sigma Chemical, St.Louis Mo. (Sigma Technical Bulletin No. 510). To determine sucrose levels, an aliquot of disk eluent was

Figure 4-1. Photograph of a half red 'Brighton' strawberry fruit which a paper filter disk, 6 mm in diameter, was placed on the surface of the fruits' central cavity.

incubated at 50° C for 2 h with 15 units per ml invertase (Sigma), to hydrolyze sucrose, and then assayed as described before for glucose. The sucrose level was determined by the difference in glucose concentrations with and without invertase hydrolysis.

4.4 Results

Fruit water potential - Fruit ψ_{aw}, as measured with *in situ* hygrometers, decreased linearly as fruit developed from 5 DAA (green stage) to 25 DAA (red), whereas $\psi_{x\text{-}press}$, determined with a pressure chamber, showed a gradual decline to a minimum value at 20 DAA (pink) and then a rise to near initial levels by 25 DAA (Fig 4-2). The calculated ψ_{as} values ($\psi_{as} = \psi_{aw} - \psi_{x\text{-}press}$) for fruit at 5, 15, 20 and 25 DAA were, -0.10, -0.25, -0.41,

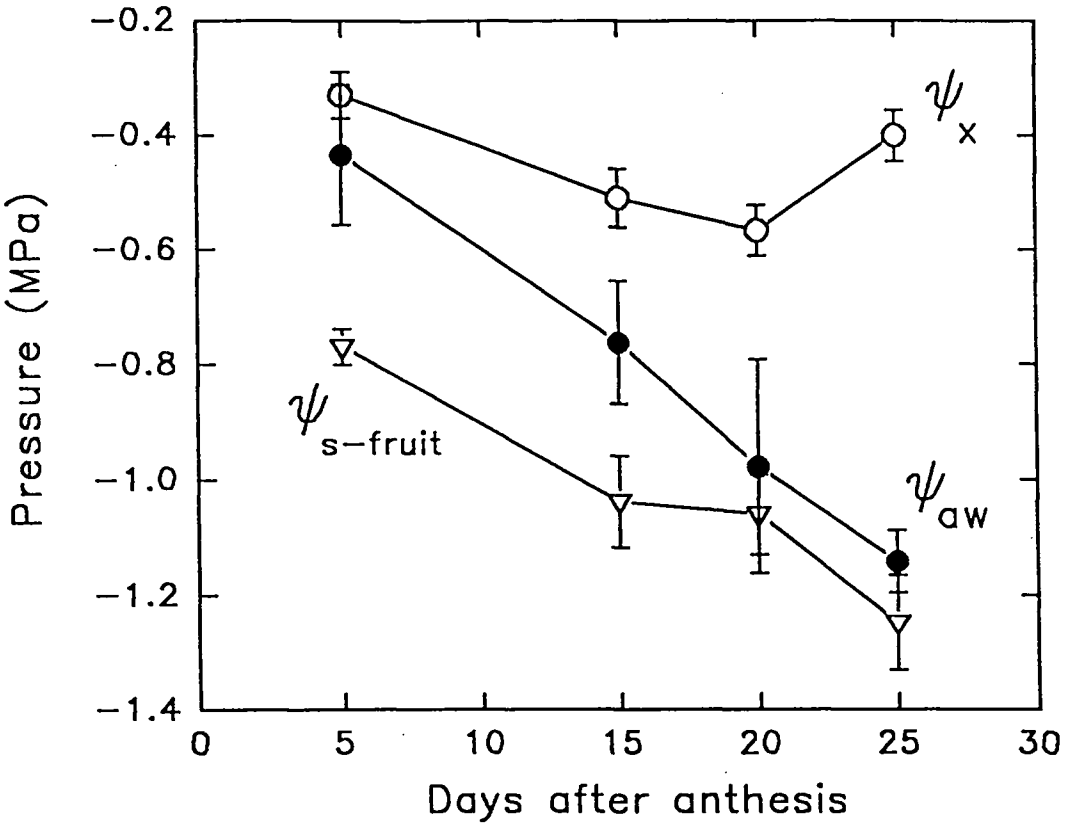

Figure 4-2. Mid-afternoon xylem pressure potential ($\psi_{x\text{-press}}$, O) determined with a pressure chamber, and apoplastic water potential (ψ_{aw}, ●) measured with *in situ* hygrometers, for a greenhouse grown primary strawberry fruit at four times during fruit development. Portions of the fruit were frozen, thawed and solute potential ($\psi_{s\text{-bulk}}$, ∇) was determined from the cell sap using a vapor pressure osmometer. Each symbol represents the mean of five fruit. Error bars represent standard error.

and -0.74 MPa, respectively. The more negative ψ_{as} values for pink and red fruit may reflect higher levels of apoplastic solutes in ripening fruit. The $\psi_{s\text{-}bulk}$ also tended to decrease with fruit development and values were similar to those of ψ_{aw} in pink and red fruit (Fig. 4-2). The calculated cell turgor decreased to near zero ($\psi_{turgor} = \psi_{aw} - \psi_{s\text{-}bulk}$) in pink and red fruit (data not shown).

Xylem exudate collection - Results from a separate xylem exudate study were similar to those described above, and are presented in Table 4-1. Compared to green and green-white fruit, red ripe fruit again showed the lowest, most negative, values of ψ_{aw}, ψ_{as}, and $\psi_{s\text{-}bulk}$, but not $\psi_{x\text{-}press}$. The solute potential of exudate ($\psi_{s\text{-}xylem}$) collected in this study is shown in Figure 4-3. Ten sequentially collected 7 ml samples were obtained from three fruit each at three stages of development by increasing chamber pressure. The $\psi_{s\text{-}xylem}$ of green fruit increased quickly to a plateau of about -0.25 MPa over an applied pressure range of 0.4 to 0.5 MPa (Fig. 4-3A). Pressure as high as 1.2 MPa was required to collect ten samples of exudate from green-white and red fruit and the $\psi_{s\text{-}xylem}$ in both stabilized at about -0.30 MPa with increasing pressure (Fig. 4-3B and 4-3C). The lack of a marked decline in the solute potential of xylem exudate as strawberry fruit matured (Table 4-1 and Fig. 4-3) is in contrast to the observed decrease in the calculated ψ_{as} (Table 4-1). The calculated ψ_{xwp} (where $\psi_{xwp} = \psi_{s\text{-}xylem} + \psi_{x\text{-}press}$) was similar in green-white and red fruit and higher in green fruit. The ψ_{xwp} was lower than ψ_{aw} in green and green-white stages, and much higher in red fruit.

The first exudate sample collected from the fruit likely contained solutes released from damaged cells at the cut surface of the fruit pedicel. The level of sugars in this

Table 4-1. Comparison of xylem exudate components to water relations of developing strawberry fruit. Xylem pressure potential ($\psi_{x\text{-}press}$) determined with a pressure chamber and apoplastic water potential (ψ_{aw}) measured with *in situ* hygrometers for single primary strawberry fruit during mid-afternoon in a greenhouse. Solute potential ($\psi_{s\text{-}bulk}$) of portions of the fruit was determined from the cell sap using a vapor pressure osmometer. Green, green-white, and red fruit stages are at 7, 15, and 25 days after anthesis. *Duncans separation of means at a level of significance of 5%, n = 3, ± standard error.

Fruit color	Fruit solute potential $\psi_{s\text{-}bulk}$	Xylem pressure potential $\psi_{x\text{-}press}$	Xylem exudate solute potential $\psi_{s\text{-}xylem}$	Total xylem water potential (calculated) ψ_{xwp}	Apoplastic water potential ψ_{aw}	Apoplastic solute potential (calculated) ψ_{as}
	MPa					
Green	-0.73 ±0.02 a*	-0.38 ±0.03 a	-0.29 ±0.01 a	-0.67 ±0.02 a	-0.50 ±0.11 a	-0.12 ±0.08 a
Green-white	-0.91 ±0.03 a	-0.58 ±0.04 b	-0.34 ±0.03 a	-0.92 ±0.03 b	-0.66 ±0.13 a	-0.08 ±0.07 a
Red	-1.20 ±0.11 b	-0.46 ±0.02 a	-0.34 ±0.01 a	-0.80 ±0.01 b	-1.15 ±0.07 b	-0.69 ±0.07 b

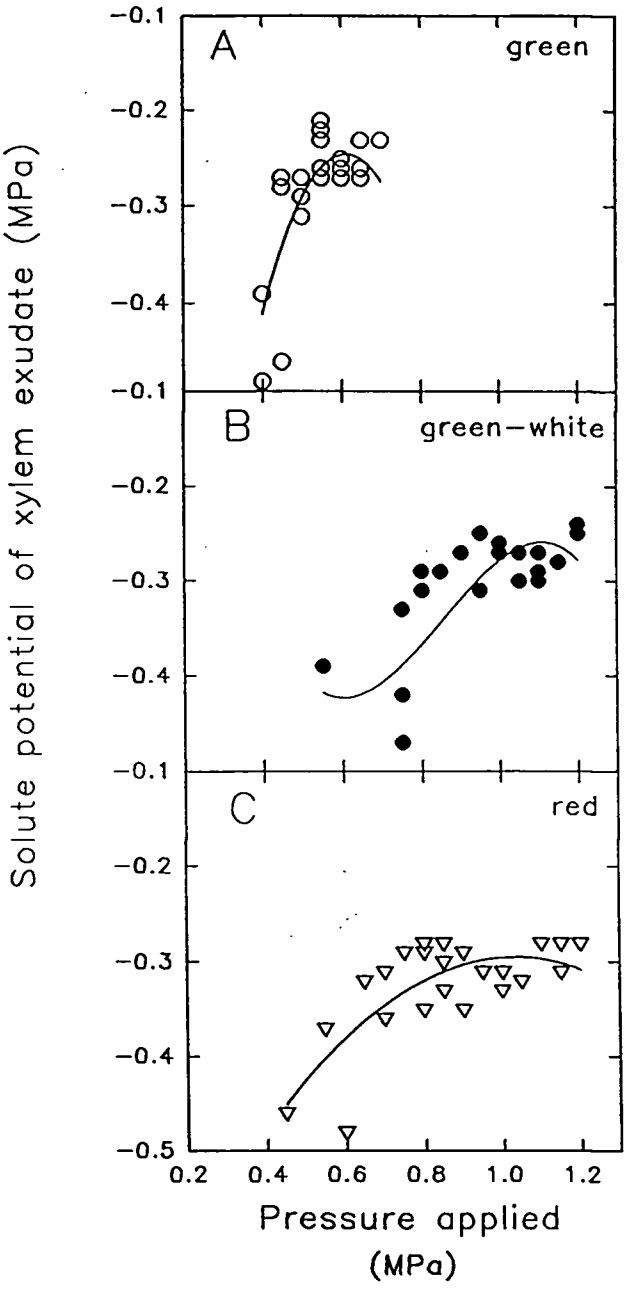

Figure 4-3. Solute potential of xylem exudate ($\psi_{s\text{-}xylem}$) collected from green, green-white, and red primary strawberry fruit in relation to pressure applied with a pressure chamber. Ten exudate samples were collected from three fruit at each developmental stage. Each symbol represents an exudate sample, with some symbols directly overlapping. Polynomial regression lines were fit for, A) green ($y = -1.62 + 4.50x - 3.67x^2$, $r^2 = 0.66$), B) green-white ($y = 0.81 - 5.02x + 6.44x^2 - 2.51x^3$, $r^2 = 0.50$), and red developmental stages ($y = -0.78 + 0.94x - 0.46x^2$, $r^2 = 0.57$).

sample was many fold higher than in subsequent samples (Fig. 4-4). However, $\psi_{s\text{-}xylem}$ was unchanged after the second disk of exudate was collected (Fig. 4-3). Based on these considerations, the second exudate sample was selected as most appropriate in attempting to characterize the apoplastic solution of the fruit. The $\psi_{s\text{-}xylem}$ value of this sample was essentially the same (about -0.3 MPa) for the three developmental stages (Table 4-1). The $\psi_{s\text{-}xylem}$ was more negative than the calculated ψ_{as} for fruit at the green and green-white stages, but less negative in red fruit (Table 4-1). At all fruit developmental stages the concentrations of glucose and sucrose in the second sample collected were each less than 1.0 mM.

The concentration of sucrose in the xylem exudate from fruit at all three developmental stages was relatively high in the initial sample, many times higher than that of glucose, and was sharply lower in subsequent samples (Table 4-2; Fig. 4-4). Glucose showed a similar, but less dramatic, pattern. The high sucrose to glucose ratio (about 5:1) in the initial sample suggests that it was not derived from strawberry fruit (receptacle) symplast, since Forney and Breen (1986) found a sucrose and glucose concentration ratio of about 1:1 in red fruit of the cultivar used here. Similarly, we found pedicels from red strawberry fruit had a sucrose:glucose ratio of 1:1 (both 1.15 mmol gFW^{-1}). The relatively high level of sucrose in the first sample of xylem exudate may reflect compartments or cell types within the pedicel, such as the phloem, which are enriched in sucrose.

Table 4-2. Glucose and sucrose in xylem exudate collected on disks described in Table 4-1. Green, green-white, and red fruit stages are at 7, 15, and 25 days after anthesis. *Duncans separation of means at a level of significance of 5%, n = 3, ± standard error.

Fruit color	Xylem exudate	
	glucose	sucrose
	mM	
Green	0.06 ±0.03 a	0.46 ±0.36 a
Green-white	0.05 ±0.01 a	0.81 ±0.17 a
Red	0.07 ±0.01 a	0.24 ±0.13 a

The levels of glucose and sucrose were low in later collected samples of xylem exudate from immature fruit (Fig. 4-4A and 4-4B). However, the sucrose level in exudate from red fruit increased markedly after the fifth sample and then appeared to reach a plateau in the last three samples (Fig. 4-4C). This rise in sucrose level was accompanied by high variability. A small increase in glucose levels in later samples was also observed. These changes may have resulted from damage of fruit or pedicel cells at high chamber pressures, however, the solutions were not visibly red as might be expected if damaged fruit cells released anthocyanins to the apoplast.

In green fruit injected with azosulfamide, the red dye was evident immediately after the first bubbles appeared at the cut end of the fruit pedicel. However, dye was never observed in the exudate of green-white and red fruit, even after ten samples were

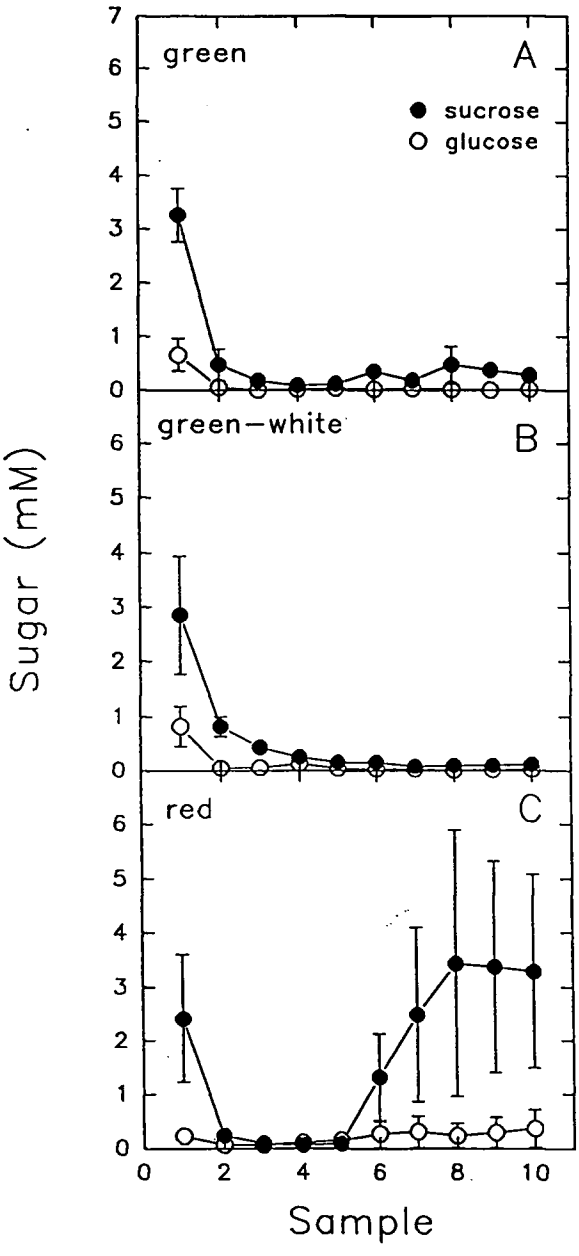

Figure 4-4. Concentrations of sucrose (●) and glucose (O) in exudate collected from strawberry fruit placed in a pressure bomb. Each symbol represents the mean of disks collected from three fruit at green (A) and green-white (B) and six different fruit at red (C) developmental stages. Error bars represent standard error.

collected and chamber pressures reached 1.2 MPa (data not shown), suggesting the development of a barrier to solute movement between the main body of the ripening fruit and pedicel xylem.

Centrifugation of tissue - No liquid was recovered from cortex tissue plugs of either green or green-white fruit when centrifuged at 30 to 4000x *g* in ten sequential steps. The result was the same whether fruit was picked in the afternoon or prior to dawn, when fruit ψ_{as} is highest (data not shown). Similarly, no liquid was recovered when receptacle tissue from pink fruit was centrifuged at a force of less than 750x *g*. However, centrifugation at six steps between 750 to 4000x *g* yielded 10-20 ml of solution after each spin. Each sample collected was pink, implying cell damage and release of anthocyanins. The solute potential of the liquid recovered by centrifugation ($\psi_{s\text{-}cent}$) was similar to the $\psi_{s\text{-}bulk}$ (Fig. 4-5A), indicating that either solute potential of the apoplastic solution is similar to that of the fruit symplast or that this procedure is destructive to strawberry fruit tissue and that much of the recovered solution was derived from damaged cells.

Centrifugation of cortex tissue from red fruit yielded 10-20 ml of red liquid at each of the seven steps from 300 to 4000x *g*. No liquid was recovered at lower centrifical forces. Again the $\psi_{s\text{-}cent}$ was similar to that of the bulk $\psi_{s\text{-}bulk}$ of the fruit (Fig. 4-5B).

Fruit cavity collection - Filter paper disks were reddish in appearance when removed from the cavity or when touched to the cut surface of fruit at pink and red stages. Infrequently, very small clumps of fruit cells adhered to the paper disk. The cavity in green-white fruit was not large enough to yield reliable samples because material from cut cells could easily contaminate the paper disks during their insertion or retrieval.

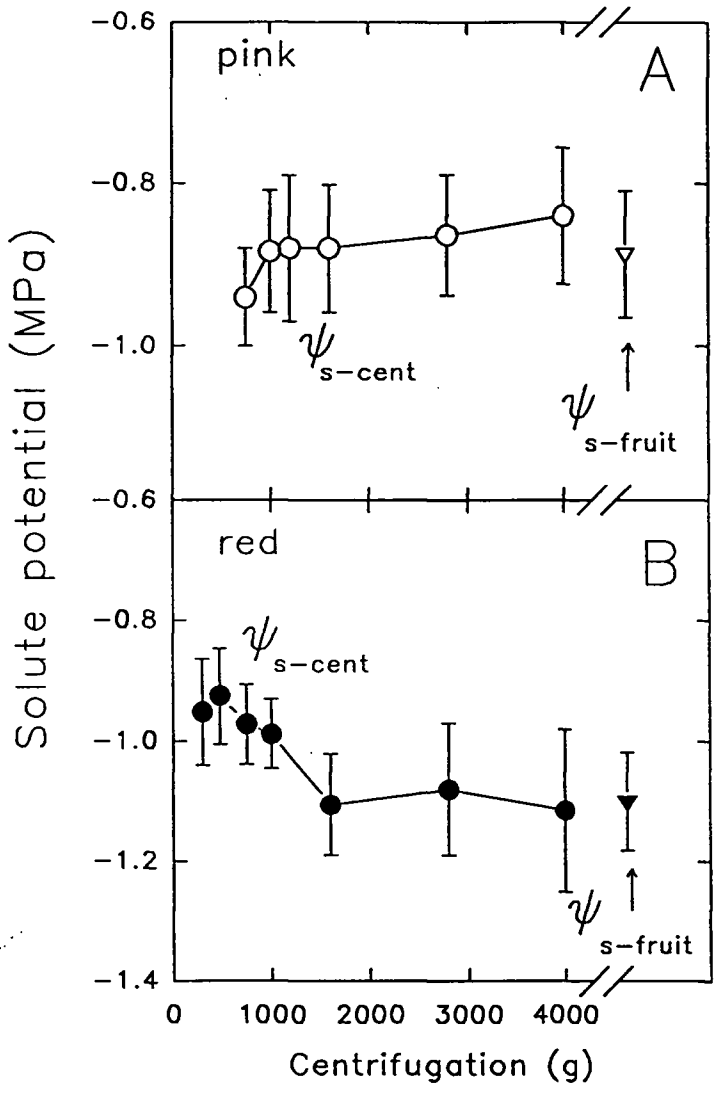

Figure 4-5. Solute potential of solution ($\psi_{s\text{-}cent}$) removed sequentially after centrifugation of strawberry fruit cylinders (1 cm diameter) and measure with a vapor pressure osmometer. The fruit cylinder was frozen at the completion of centrifugation, thawed and solute potential ($\psi_{s\text{-}bulk}$) was determined from the cell sap using a vapor pressure osmometer. Each symbol represents the mean of three fruit. Error bars represent standard error.

A disk usually displayed a wet sheen after 15 or 30 min in the fruit cavity. Occasionally, however, a disk placed in a cavity of a red fruit accumulated very little liquid and was discarded. The cause of poor uptake of solution by a disk is unknown, but it may indicate that the quantity of apoplastic solution in a strawberry fruit fluctuates in response to environment or physiological factors. Based on a comparison of collected disks with a standard curve of disk weight vs. volume of added water, disks retrieved from a fruit cavity after 15 or 30 min usually contained about 5 ml of solution, whereas those placed on the cut fruit surface held approximately 7 ml. There was no difference in the measured osmolarity of 5 or 7 ml aliquots of salt solution standard using the vapor pressure osmometer, indicating that osmolarities measured with volumes collected from the fruit cavity and cut surface are directly comparable.

The solute potential of the solution from the cavity ($\psi_{s\text{-cavity}}$) of red fruit was significantly higher than that recovered from the surface of cut receptacle tissue ($\psi_{s\text{-surface}}$), but not significantly different from the bulk $\psi_{s\text{-bulk}}$ (Table 4-3). In pink fruit there were no significant difference in the values of these parameters, although $\psi_{s\text{-cavity}}$ was again highest. Stage of fruit ripeness did not have a significant effect on solute potential. Similar concentrations of glucose and sucrose were recovered from the cavity of pink and red fruit, ranging from 47 to 62 mM (Table 4-3). These concentrations were comparable or somewhat lower than the levels of glucose and sucrose in the liquid collected from the cut surface of the fruit.

Results of the four methods used in this study to determine solute potential of the apoplastic solution of strawberry fruit, by either attempting to obtain samples of the

solution or indirectly by measuring associated parameters, are summarized in Table 4-4. These values of solute potential may not be directly comparable since $\psi_{s\text{-}fruit}$ was not constant for a given fruit developmental stage. The solute potential of the apoplast may be linked with that of the bulk fruit, therefore, to correct for the variation in $\psi_{s\text{-}bulk}$, apoplastic solute potential obtained by the various methods is also expressed as a ratio to the measured $\psi_{s\text{-}bulk}$. Based on the data shown in Table 4-3, the apoplast of ripening strawberry fruit contained high levels of solutes which could enhance phloem transport by the steepening ψ_{aw} gradient between the leaves and fruit. The low apoplastic solute levels in young fruit (see Table 4-1 and Fig. 4-1) indicate that they do not play a role in the establishment of a ψ_{aw} gradient to influence transport to developing fruit.

4.5 Discussion

The estimation of ψ_{as} from the difference between ψ_{aw} and $\psi_{x\text{-}press}$, a relatively fast and easy method, indicated increasing levels of solute in the apoplast as fruit ripened. Based on solute potential values, the concentration of apoplastic solute in red fruit, as estimated by this method, is over twice that found in the xylem exudate, but about 67% of that determined from directly sampling the fruit cavity or by centrifugation (Table 4-4). The validity of $\psi_{x\text{-}press}$ measurements made with a pressure chamber to represent the total water potential of a tissue has been called into question. Recently, Zimmermann's group

Table 4-3. Solute potential and sugar concentrations of solution collected on paper filter disks from the strawberry fruit cavity and cut surface. Solute potential of solution collected from the cavity ($\psi_{s\text{-cavity}}$) or cut surface ($\psi_{s\text{-surface}}$) or from cell sap collected from portions of the fruit ($\psi_{s\text{-bulk}}$) were determined using a vapor pressure osmometer. Glucose and sucrose concentrations were determined colormetrically from liquots of solution collected. *Duncans separation of means at a level of significance of 5%, n = 10. ** Calculated based on 7 and 5 ml collected from the cut surface and cavity of fruit, respectively. ± standard error.

Site	Solute Potential (MPa)	Glucose (mM)	Sucrose (mM)
Pink fruit stage			
Cavity ($\psi_{s\text{-cavity}}$)	-1.01 ±0.03 a*	49.8** ±7.3 a	54.4 ±13.7 a
Cut surface ($\psi_{s\text{-surface}}$)	-1.12 ±0.05 abc	67.2 ±14.2 ab	52.8 ±8.4 a
Fruit bulk ($\psi_{s\text{-bulk}}$)	-1.13 ±0.03 abc	-	-
Red fruit stage			
Cavity ($\psi_{s\text{-cavity}}$)	-1.05 ±0.06 ab	62.1 ±9.3 ab	46.8 ±15.8 a
Cut surface ($\psi_{s\text{-surface}}$)	-1.21 ±0.06 c	85.7 ±8.9 b	77.6 ±18.9 a
Fruit bulk ($\psi_{s\text{-bulk}}$)	-1.19 ±0.04 bc	-	-

Table 4-4. Summary of apoplastic solute potential (ψ_{as}) determined by four methods and the ratio of ψ_{as} to solute potential ($\psi_{s\text{-}bulk}$) in strawberry fruit at two developmental stages. Methods used: [z]calculation based on $\psi_{as} = \psi_{aw} - \psi_{x\text{-}press}$ from Fig. 2, [y]xylem exudate from second disk collected via pressure bomb from Table 1, [x]solution collected via centrifugation of tissue cylinder from Fig. 5, and [w]solution collected from the fruit cavity from Table 2. [v]xylem exudate from second disk and ratio for green-white fruit. Color of solution was the same in either pink or red stage when noted.

	Method			
Fruit Stage	Calculation[z] ψ_{as}	Xylem exudate[y] $\psi_{s\text{-}xylem}$	Centrifugation[x] $\psi_{s\text{-}cent}$	Cavity[w] $\psi_{s\text{-}cavity}$
	MPa			
Pink	-0.42	-0.34[v]	-0.89	-1.01
Red	-0.74	-0.34	-1.00	-1.05
Color of solution	-	not colored	pink to red	pink to red
	Ratio			
	$(\psi_{as}/\psi_{s\text{-}bulk})$	$(\psi_{s\text{-}xylem}/\psi_{s\text{-}bulk})$	$(\psi_{s\text{-}cent}/\psi_{s\text{-}bulk})$	$(\psi_{s\text{-}cavity}/\psi_{s\text{-}bulk})$
Pink	0.39	0.37[v]	0.95	0.89
Red	0.59	0.29	0.86	0.88

(Balling and Zimmermann, 1990; Zimmermann et al., 1993) found that xylem tension measured with a pressure probe was much lower than that determined with a pressure chamber, and concluded that the use of a pressure chamber often leads to overly negative $\psi_{x\text{-}press}$ measurements. If $\psi_{x\text{-}press}$ was underestimated (i.e., more negative) in ripening strawberry fruit in this present study, ψ_{as} may be more negative than that calculated and closer to values from the centrifugation and cavity methods. Since $\psi_{x\text{-}press}$ is a component

in the calculation of ψ_{xwp}, its underestimation would make ψ_{xwp} similar to ψ_{aw} values in less mature fruit.

The exudate collected from the xylem of red fruit had the highest solute potential compared to samples collected directly from the fruit cavity and by centrifugation, with the apparent concentration of solutes in xylem exudate only a third that in solutions collected via these other methods (Table 4-4). For exudate to be pushed from the cut surface of the pedicel, the chamber pressure must surpass the fruit $\psi_{x\text{-}press}$ and water must move into the xylem from other tissues. Jachetta et al. (1986) suggested that exudate collected from the cut petiole of a leaf by incremental rises in overpressure would be increasingly diluted by water forced through membranes of parenchyma cells of leaf veins some distance from the cut surface. Canny (1993), on the other hand, has suggested that overpressure could instead immediately force water from parenchyma cells near the cut surface causing a several fold dilution of even the initial exudate sample collected. In the case of the strawberry fruit, even the first exudate sample collected may be diluted by water derived from cells proximal to the cut surface of the pedicel.

The recovery of injected dye from the green fruit and the inability to collect it from the xylem of green-white and red fruit is consistent with the development of a barrier to solute movement between the main body of the ripening strawberry fruit and the xylem. Possibly, the higher chamber pressures needed to obtain ten samples of xylem exudate from green-white and red fruit, compared to green fruit (see Fig. 4-3), are a consequence of an anatomical barrier to solute movement developing as strawberry fruit mature. Similarly, the extremely low levels of sucrose and glucose, generally many

orders of magnitude less than that recovered from the fruit cavity, and the lack of any red pigment in the xylem exudate indicates that apoplastic solutes in ripening strawberry fruit are somehow isolated from the pedicel xylem and can not be retrieved by the pressure chamber method.

Several types of anatomical barriers have been described that prevent the back flow of solutes into the xylem from the sink apoplast. Xylem discontinuity in grape (Findlay et al., 1987) and tomato (Lee, 1989) pedicels isolate these fruit from the rest of the plant, thereby preventing the movement of solutes back into the xylem. Although a fruit-pedicel abscision zone does not form in strawberry, ripe fruit of this cultivar are easily detached at the calyx, possibly reflecting a weakening in the vasculature in this region. Suberized and lignified bundle sheath cell walls have been reported by Welbaum et al. (1992) that obstructed solute diffusion from the sugar storage region in sugarcane to the vascular bundles which prevented the backflow of sucrose into the xylem. Canny (1993) has also identified the presence of semi-permeable barriers to solute movement in the xylem of leaves. Similar barriers to solute movement may be present in ripening strawberry fruit.

The similarity in solute potential values of the liquid recovered by centrifugation of tissue from pink and red fruit ($\psi_{s\text{-}cent}$) to that of $\psi_{s\text{-}bulk}$ indicates that the apoplastic concentration of solutes is relatively high in ripening fruit, and/or that this procedure damaged cells and their released solutes were recovered in the solution collected. The inability to collect liquid by centrifugation from green or green-white fruit tissue may represent the increased ability of these tissues to hold water prior to the events that

accompany fruit softening. Extreme hydration of the middle lamella and wall matrix material has been reported in ripening strawberry fruit (Knee et al., 1977). Some solutes were likely released from cut and damaged cells of the cylinder of fruit tissue placed in the centrifuge tube. Fruit tissue plugs were not rinsed prior to centrifugation due to concern for diluting the apoplast solution (Welbaum and Meinzer, 1990). It is presumed that substances released from damaged pink or red strawberry fruit cells were reabsorbed by the tissue or recovered in the initial quantities of liquid collected. Since the liquid obtained by centrifugation was red, this method may have been too harsh for fragile strawberry fruit tissue, causing cell damage and leakage of vacuole-stored anthocyanins and other solutes to the apoplast. Conversely, the release of anthocyanins and solutes into the apoplast may be an inherent consequence of ripening in strawberry fruit. Lang and During (1991) obtained red exudate from the xylem of a grape berry with pigmented flesh and concluded that this resulted from a loss of compartmentation in the fruit at the onset of ripening.

Due to the pith cavity that forms in the center of ripening fruit, the strawberry offers an opportunity for sampling the apoplastic solution of an attached organ. The relatively low solute potential ($\psi_{s\text{-cavity}}$) and the red color of the disks retrieved from the cavity of pink and red fruit strongly suggest that fruit cortex cells had released symplastic solutes, including sugars and anthocyanins, to the apoplast. Although disks retrieved from a cavity occasionally contained clumps of red cells, these were not responsible for the general red color of the disks.

Both sucrose and glucose were present at concentrations of about 50 mM in the solution collected from the cavity of pink and red fruit (Table 4-3). Fructose, which we did not measure, is likely present in the apoplast at levels similar to that of glucose, since these hexoses are near a 1:1 ratio in ripening 'Brighton' fruit (Forney and Breen, 1986). John and Yamaki (1994) recently used compartmental analysis to estimate sugar pools in the vacuole, cytoplasm, and free space in tissue plugs from ripening strawberry fruit. Using this indirect method, they calculated the total sugar concentration in the apoplast of light green and slightly red fruit as 167 and 217 mM, respectively. The latter value is in rough agreement with our estimated total concentration of 150 mM for sucrose and hexoses in the apoplast of red strawberry fruit. John and Yamaki (1994) calculated that the apoplastic region in strawberry fruit accounts for only 13% of the total sugar in the fruit. Using the van't Hoff equation (e.g. ψ_s = -Conc.RT at 20°C) and a concentration of 150 mM, the total ψ_s of sucrose, glucose, and fructose in the apoplast of a red fruit can account for about -0.37 MPa, or about 35%, of the $\psi_{s\text{-cavity}}$ of -1.05 MPa (from Table 4-3).

Sucrose in the apoplast of ripening strawberry fruit, as recovered in the cavity method, may have been unloaded from the phloem into the apoplast. Since neither glucose nor fructose are transported in the phloem of strawberry (Forney and Breen, 1986), glucose detected in the recovered solution could have resulted from sucrose hydrolysis in the apoplast. Poovaiah and Veluthambi (1985) have reported the presence of invertase activity in a cell wall fraction from developing strawberry fruit. Additionally, both glucose and sucrose may have leaked from fruit cells into the apoplast. All three sugars are likely retrieved from the apoplast by surrounding cells. Based on

uptake kinetics with tissue from green-white strawberry fruit (Forney and Breen, 1986), both saturable and linear uptake systems may be involved in the uptake of sucrose from the apoplast, with the linear system predominating at the sucrose concentration detected in red fruit. If apoplastic glucose and sucrose are each at 50 mM, the rate of glucose uptake by its saturable uptake system would be over two fold greater than the uptake of sucrose. However, if membrane integrity decreases greatly in a strawberry fruit as it develops beyond the green-white stage, the rate of net uptake of sucrose and hexose from the apoplast by fruit cells may be appreciably different than that described for less mature fruit tissue.

In conclusion, xylem exudate from strawberry fruit did not allow sampling of the apoplast, while other methods yielded more reliable and comparable estimates of ψ_{as}. The apoplast of ripening fruit contained high levels of solutes, most of which were not sugars, possibly enhancing phloem delivery of water and assimilate to the fruit. The relatively low level of apoplastic solutes in young fruit suggests that they do not play an important role in transport early in development. The linear, and to a lesser extent the saturable, component of sucrose uptake may be functioning in the ripening fruit apoplast at the sucrose concentration (50 mM) measured.

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CHAPTER 5

THE EFFECT OF CELL TURGOR ON SUGAR UPTAKE IN STRAWBERRY FRUIT CORTEX TISSUE

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5.1 Abstract

A reduction in cell turgor has been shown to stimulate sugar uptake in several plant sink tissues and it may regulate the import of assimilate into the sink apoplast, as well as maintain cell turgor. To determine whether cell turgor influences sugar uptake by strawberry (*Fragaria* x *ananassa* Duch. cv. Brighton) fruit cortex tissue, disks were cut from greenhouse-grown primary fruit at the green-white stage of development and placed in buffered incubation solutions containing either mannitol or ethylene glycol as an osmoticum. Cell turgor of fruit disks was calculated from the difference between the water potential of bathing solution and tissue solute potential after incubation at various osmolarities. Cell turgor increased when tissue disks were placed into mannitol incubation solutions more dilute than the water potential of fresh tissue (about 415 mOsmol kg^{-1}). The rate of uptake of [^{14}C]-sucrose or [^{14}C]-glucose decreased as osmolarity of the incubation solution increased, i.e. as cell turgor declined. Cell turgor and the rate of [^{14}C]-sucrose uptake were unaffected when rapidly permeating ethylene glycol was used as an osmoticum. A decrease in cell turgor reduced both the V_{max} of the saturable (carrier mediated) kinetic component of sucrose uptake and the slope of the linear (diffusional) component. The sulfhydryl binding reagent p-chloromercuibenzenesulfonic acid, an inhibitor of the plasma membrane sucrose carrier, strongly inhibited only the saturable component of sucrose uptake. Increased uptake of the nonmetabolizable sugar, O-methyl-glucose, at high turgor was similar to that of glucose, indicating that carrier activity was influenced by cell turgor, not cell metabolism. Turgor did not influence efflux of [^{14}C]-sucrose from disks and had no

effect on cell viability. Strawberry fruit cells do not possess a sugar uptake system that is stimulated by a reduction in turgor.

5.2 Introduction

How sink regions in plants regulate the unloading of solutes from the phloem is poorly understood. Sugar and other solutes are translocated in the phloem by a passive bulk flow down a gradient of hydrostatic pressure from source to sink regions. In sink tissues, solutes may be unloaded from the phloem into the apoplast (the whole continuity outside the plasma membrane) where they are taken up by sink cells, or they may be unloaded symplastically (cell to cell via plasmodesmatal connections), or by a combination of these two systems (Oparka, 1990; Patrick, 1990). Removal of solutes from sites of unloading could steepen the hydrostatic gradient between source leaves and sink tissues (Wolswinkle, 1990; Patrick, 1994a, b).

The rate of transport of assimilate to sink regions may be influenced by turgor-sensitive uptake of solutes from the sink apoplast. Lang and Thorpe (1986) have postulated that high levels of apoplastic solutes promote the removal of water from the phloem, reducing its turgor, and presumably that of sink cells, and increasing the efflux of translocated solutes into the apoplast for subsequent uptake by sink cells. They demonstrated that steepening the water potential gradient between the shoot and root of *Phaseolus vulgaris* L., by bathing the root in a solution containing a non-permeating osmoticum, increased assimilate flow to the root. One way by which transport of sugars and other solutes to a sink region with high apoplastic solutes could be maintained is

through a mechanism in which declining turgor stimulates solute uptake by sink cells. Apoplastic water would move into these cells as they accumulated solutes, allowing continued water and assimilate import to the region. A reduction in cell turgor, by increasing the concentration of a nonpermeating osmoticum in a bathing solution, was shown to increase apoplastic sucrose uptake in tissues of sugar beet taproot (Wyse et al., 1986) and potato tuber (Oparka and Wright, 1988a, b), as well as in leaves (Daie and Wyse, 1985) and young cotyledons of *Phaseolus* (Patrick, 1994a).

Turgor-sensitive sugar uptake from the apoplast of sink tissues may also represent a point of regulation in maintaining cell turgor. Maintenance of cell turgor through the accumulation of solutes within cells, or osmoregulation, is often a strategy by which plants tolerate salt or water stress (Morgan, 1984). Positive cell turgor is also required for plant growth (Meyer and Boyer, 1981).

Sucrose, the major sugar translocated in strawberry (Forney and Breen, 1985a), may be unloaded into the apoplast of the fruit (Forney and Breen, 1986). Levels of apoplastic solutes increase during strawberry fruit development, with red fruit having apoplastic solute potentials as low as -1.0 MPa and sucrose and glucose concentrations at approximately 50 mM each (Chapter 5; Pomper and Breen, 1995). Forney and Breen (1986) reported that uptake of sucrose *in vitro* by tissue from strawberry fruit at the white-pink stage displays biphasic kinetics, with a saturable component predominant at low concentrations (< 25 mM) and a linear component at higher concentrations. Fructose uptake showed a similar pattern. Uptake of glucose was more rapid and its kinetics revealed only the first component, which saturates near 200 mM. The influence of

incubation solution osmolarity (thereby cell turgor) on sugar uptake by strawberry fruit tissues has not been examined.

Our longterm goal is to understand the mechanism of sugar transport and accumulation in strawberry fruit. The objective of the present study was to determine whether the rate and kinetics of uptake of sucrose and glucose *in vitro* are influenced by turgor in fruit tissue disks.

5.3 Materials and methods

Plant material - Plants of the culitvar Brighton, a day-neutral strawberry (*Fragaria* x *ananassa* Duch.), were grown in 3.8-l pots in a mixture of equal parts of soil, coarse sand, peat (v/v/v) in a greenhouse that was maintained at 30/18°C (day/night), and where high-pressure sodium lamps (400 watt, Energy Techtonics Horticulture Lighting, York, Penn., USA) (175 ±25 mmol m^{-2} s^{-1}) provided a 16-h photoperiod. Primary flowers were pollinated with a small paint brush, tagged, and all competing flowers or flower buds were removed from an inflorescence.

Fruit tissue water relations - Disks of fruit tissue were prepared as described by Forney and Breen (1986). Briefly, disks (1.3 x 7.0 mm) were cut from the cortex of primary fruit at the green-white stage of development (15-20 days after anthesis), pooled and preincubated for 1 h in 10 ml of buffer solution at selected osmolarities. Disks were then distributed into 50-ml Erlenmeyer flasks containing 5 ml of buffer solution at the treatment osmolarity. Fruit disks from pink and red fruit disintegrated during incubation and were not used in this study. The buffer solutions consisted of 50 mM $CaCl_2$, 20 mM

citric acid (pH 5.0), and either mannitol or ethylene glycol as an osmoticum unless otherwise noted. The buffer solution was approximately 180 to 190 mOsmol at preparation, prior to the addition osmoticum. Using strawberry fruit disks, Forney and Breen (1986) found that 50 mM $CaCl_2$ maintained the maximum respiration rate and a pH optimum of 5.0 with this buffer yielded the highest uptake of [^{14}C]-sucrose. Mannitol, which permeates slowly, was used to alter cell turgor, whereas rapidly permeating ethylene glycol was used to change cell osmolarity without modifying cell turgor (but see Cram, 1984). Inclusion of 100 mM mannitol in buffer solutions increased osmolarity by about 100 mOsmol. During preincubation and uptake studies, flasks containing disks were placed in a gyratory water bath shaker (80 rpm) at 25°C for 1 or 4 h.

After incubation, tissues were frozen for 1 h at -80°C, thawed, and the cell sap was obtained by centrifugation at 4°C (3,000 *g* for 5 min). After 5 additional min at room temperature, sap and incubation solution osmolarity were determined with a vapor pressure depression osmometer (Wescor 5100 C, Wescor Inc., Logan, UT). The solute potential can be calculated using the vant'Hoff equation: solute potential = -CRT, where C is the molality of the solution or osmolarity (moles of solute kg H_2O^{-1}), R is the gas constant (0.00831 kgMPa mol^{-1} K^{-1}), and T is the absolute temperature (K) (Salisbury and Ross, 1992). The measured osmolarity of cell sap and incubation solution was transformed to tissue solute potential and solution water potential respectively by multiplying mOsmol kg^{-1} with -2.48 MPa (Welbaum and Meinzer, 1990). The average

turgor was calculated as the difference between tissue solute potential and incubation solution water potential (Li and Delrot, 1987).

Water potential of fresh unincubated fruit tissue was determined by two methods: First, disks (1.3 x 7.0 mm) were cut from fruit, rinsed for 3 s with distilled water, blotted dry, weighed, placed into incubation solutions at various mannitol osmolarities for 1 h, and reweighed. The solution osmolarity where there was no change in tissue weight was considered equivalent to the tissue water potential (Oparka and Wright, 1988b). Second, disks (4 x 1 mm) were cut from fruit cortex tissue and placed directly into the vapor pressure depression osmometer chamber, osmolarity was measured and converted to tissue water potential. Additional unincubated cortex tissue was frozen for 1 h at -80°C, thawed, cell sap obtained by centrifugation, and osmolarity was measured and converted to tissue solute potential as described above.

Sugar uptake - The uptake of [^{14}C]-sugars by tissue disks from buffer solutions containing [^{14}C]-sucrose, [^{14}C]-glucose, [^{14}C]-mannitol (Amershine Co., Arlington Heights, IL), or 3-O-methyl-[^{14}C]-glucose (O-methyl-glucose; Sigma) at 3.33 MBq ml^{-1} was examined at various osmolarities. As described by Forney and Breen (1986), disks (1.3 x 7.0 mm) were cut, weighed and preincubated for 1 h in buffer solutions containing mannitol, sorbitol or ethylene glycol at a selected treatment osmolarity. Disks were then placed into buffer solutions that included a [^{14}C]-labeled sugar (10 mM) and incubated for 2.5 h. Sugar uptake into strawberry fruit tissue disks is nearly constant for up to 4 h (Forney and Breen, 1986).

After incubation, disks were rinsed (3 x 10 min) with ice-cold incubation solution to remove [^{14}C]-sugars from the cell apoplast, extracted overnight with 80% ethanol at 60°C, and then taken to dryness in an oven. The dry radioactive extract was resuspended in 1 ml of water, 10 ml of aqueous counting fluor (ReadyGel, Beckman, Palo Alto, CA) was added, and the amount of ^{14}C was determined with a Beckman LS 7000 scintillation counter. Only the ethanol soluble fraction is routinely reported, since less than 20% of radioactivity was contained in the ethanol insoluble portion and total ^{14}C accumulation was found to follow the same pattern as in the ethanol-soluble fraction (unpublished data of Pomper and Breen).

The effect of turgor on uptake kinetics for sugars was also studied using a range of [^{14}C]-sugar concentrations (1, 5, 10, 25, 50, 75, 100 mM) at high (455 mOsmol kg^{-1}) and low (265 mOsmol kg^{-1}) solution osmolarities. Mannitol levels were adjusted so that a given osmolarity was maintained at all [^{14}C]-sugar concentrations. Incubation solutions at 265 mOsmol kg^{-1} maintained disks at a positive turgor (based on disk turgor data in Fig. 5-1), whereas tissues were near zero turgor in solutions at 455 mOsmol kg^{-1}. Kinetic analysis was preformed using Lineweaver-Burk plots. Where two kinetic components were present, the first order rate coefficient k was calculated as the slope of the linear regression equation for the total sugar uptake above about 25 mM substrate concentrations. The contribution of the linear component to total sugar uptake was estimated as k multiplied by the substrate concentration. The linear component was subtracted from the total uptake to give rates for the saturable component.

Addition of inhibitor - The sulfhydryl binding reagent p-chloromercuribenzenesulfonic acid (PCMBS) is an inhibitor of the plasma membrane sucrose carrier (Giaquinta, 1976). In some experiments, disks were preincubated for 45 min in buffer containing 2 mM PCMBS (Sigma) but lacking sucrose (Wyse et al., 1986; Wright and Oparka, 1989), at the selected treatment osmolarities. Disks were then rinsed with PCMBS-free buffer (3 x 10 min) and uptake of [^{14}C]-sucrose (3.33 MBq ml^{-1}) was studied using a range of ^{14}C-sucrose concentrations at high (455 mOsmol kg^{-1}) and low (265 mOsmol kg^{-1}) osmolarities.

Sucrose efflux - Efflux (or exchange) of ^{14}C from disks preloaded with [^{14}C]-sucrose was examined at high and low cell turgor by incubation in 265 and 455 mOsmol kg^{-1} solutions, respectively. The half-life for exchange of sugar from the vacuole and cytoplasm was estimated by the compartmentation method (Macklon, 1976; Wyse et al., 1986). Disks were preloaded with [^{14}C]-sucrose as described in the uptake experiment and then rinsed (2 x 4 min) at 25°C with nonradioactive buffer solutions at treatment osmolarity. Efflux of ^{14}C from 10 disks was monitored for 150 min by collecting rinses, drying the liquid down, resuspending in 1 ml of water, adding 10 ml of aqueous counting fluor, and then measuring radioactivity with a scintillation counter. At the end of 150 min, disks were extracted overnight with 80% ethanol and radioactivity of the extract was determined. The contribution of efflux from the vacuole, determined from the regression analysis, was subtracted from the data to determine the contribution of efflux from the cytoplasm. Regression analysis was completed using the highest r^2 regression fit for each compartment.

Cell viability - Phenolic efflux (based on Redmann et al., 1986) was examined by rinsing disks for 5 s, then placing them into buffer solutions containing mannitol at osmolarities of 150, 310 and 610 mOsmol kg^{-1}. Solution absorbance at 280 nm was measured after 3 h of incubation. Solutions containing disks were then frozen at -80°C for 1 h, thawed, and absorbance was measured to determine the total phenolics within disks in order to calculate the percentages that leaked out during incubation.

Disks were also incubated in mannitol solutions of 150, 310 and 610 mOsmol kg^{-1} for 2 h, then the solution was spiked with Neutral Red (0.1% w/v), and after 30 min disks were placed into 0.05 M NaOH for 1 min and then put on slides under coverslips (Stadalman and Kinzel, 1972). Disks were evaluated using a blind draw of randomly numbered slides. An area (50 mm^2) of parenchyma cells on each disk was picked randomly in the first cell layer underneath cells damaged from cutting the disks. Neutral Red accumulation in the cell vacuole indicates a living cell. Viability of tissue was reported as the percentage of cells in the outermost intact cell layer that stained red.

5.4 Results

Fruit tisssue water relations - Water potential of fresh tissue disks was equivalent to about 415 mOsmol kg^{-1} (or -1030 kPa); this is shown by the gain or loss of fresh weight, respectively, in mannitol solutions below and above this osmolarity (Fig. 5-1A). Increasing solution osmolarity with ethylene glycol did not cause a decrease in disk fresh weight, indicating that this osmoticum, in contrast to mannitol, freely permeated fruit cell membranes (Fig 5-1A).

Using the vapor pressure osmometer method, water potential of fresh fruit was calculated at -1007 kPa (±86 SE, n = 10) or approximately 430 mOsmol kg^{-1}, and was thus similar to the water potential determined from the change in fresh weight. Tissue solute potential of fresh fruit was measured at -1065 kPa (±126 SE, n = 10). Therefore, the mean turgor of cells in green-white fruit was 58 kPa (water potential - solute potential = turgor) [-1007 kPa - (-1065 kPa) = 58 kPa] with a range of 0 to 100 kPa.

Cell turgor increased when tissue disks were incubated in mannitol solutions at osmolarities below 400 mOsmol kg^{-1}, which is equivalent to the water potential of fresh fruit tissue (Fig. 5-1B). Disks reached equilibrium with the incubation solution within 1 h; therefore, incubation for 4 h showed little change in turgor (Fig. 5-1B). The calculated turgor of disks increased and showed great variability in solutions from 400 to 700 mOsmol kg^{-1}. Gelling of the cell sap was observed after disks incubated in this osmolarity range were frozen and thawed; this probably caused unreliable osmolarity readings. Since tissue disks lost fresh weight at osmolarities greater than about 400 mOsmol kg^{-1} (Fig. 5-1A), the tissue would have experienced a loss of turgor rather than an increase (Fig. 5-1B).

When disks were incubated in solutions containing ethylene glycol from 165 to 665 mOsmol kg^{-1}, cell osmolarity increased but cell turgor remained at approximately 100 kPa after 1 h of incubation (data not shown). No gelling of cell sap was observed with a high osmolarity of ethylene glycol.

Sugar uptake - The rate of uptake of [^{14}C]-sucrose at 10 mM decreased with increasing osmolarity of the incubation solution (r^2= -0.61; Fig. 5-2A), showing a linear relationship

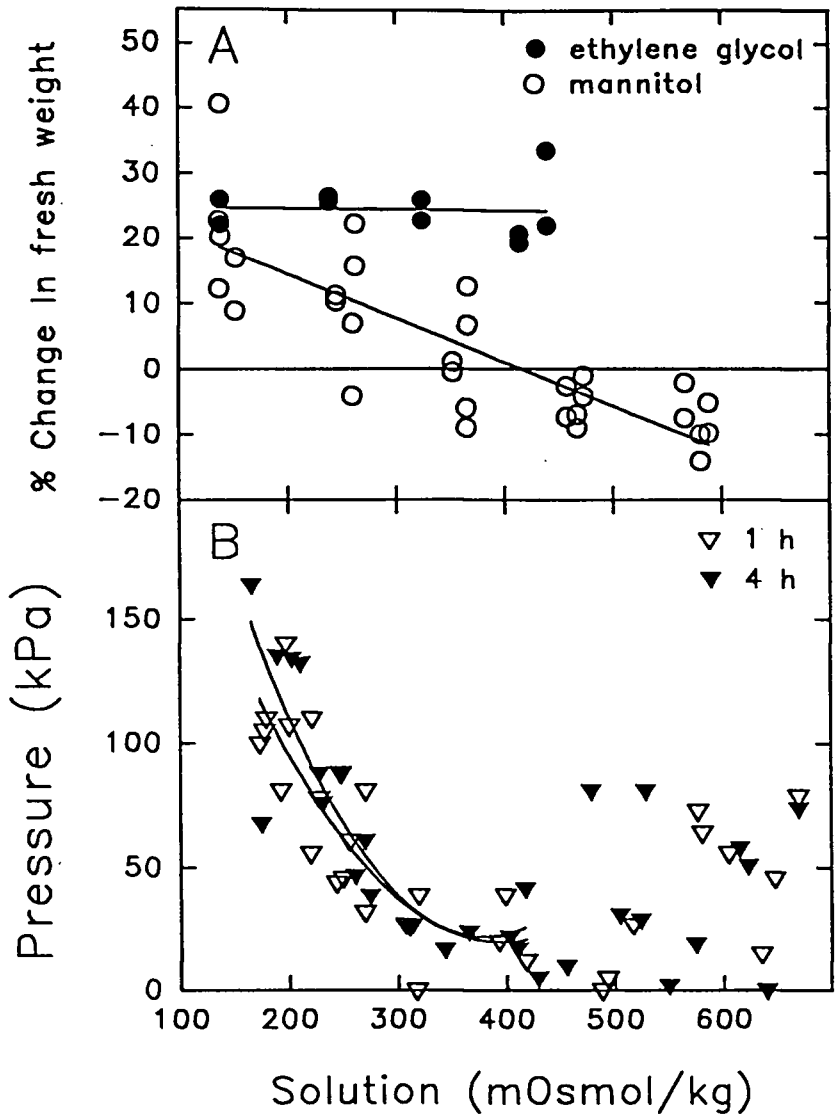

Figure 5-1. A, Percent change in fresh weight of tissue disks from green-white strawberry fruit after 1 h of incubation in solutions at various osmolarities containing mannitol (O; $y = 27.846 - 0.067x$, $r^2 = 0.76$) or ethylene glycol (●). Each symbol represents a tissue disk. Disks from five fruit were used in this experiment. B, Turgor pressure in disks, calculated by using the data in Fig. 2A, after 1 h (▽; $y = 329.370 - 1.570x + 0.002x^2$, $r^2 = 0.71$) and 4 h (▼; $y = 417.273 - 2.089x + 0.002x^2$, $r^2 = 0.77$) in incubation solutions at various osmolarities containing mannitol. The variable and high values of turgor pressure at osmolarities greater than 400 mOsmol kg^{-1} were considered artifacts due to gelling of the cell sap. Data presented is from three experiments, with each symbol the mean of five disks or a sample of incubation solution.

with the calculated cell turgor (r^2= 0.59; Fig 5-2B). Similar results were obtained for uptake of [^{14}C]-sucrose with sorbitol or polyethylene glycol 400 instead of mannitol as the osmoticum (data not shown). Uptake of [^{14}C]-glucose (10 mM) showed a negative linear relationship with incubation solution osmolarity (r^2 = -0.74) and a positive, albeit less strongly, linear relationship with cell turgor (r^2 = 0.49; Fig. 5-2A, 5-2B). The rate of uptake of [^{14}C]-sucrose at 100 mM showed a similar decrease with increasing osmolarity of the incubation solution (data not shown). The observed decrease in uptake of both glucose and sucrose with a decline in turgor indicates that turgor effects on sugar uptake were of a general character and not sugar specific. When ethylene glycol was used as an osmoticum, sucrose uptake was maintained at approximately 0.45 mmol [g fresh weight]$^{-1}$ h^{-1} for the range of 165 to 665 mOsmol kg^{-1} (data not shown). The inhibitory effect of low cell turgor on sucrose uptake was more evident at higher sucrose concentrations, suggesting that turgor altered uptake kinetics. As previously reported by Forney and Breen (1986), a saturable component following Michaelis-Menten kinetics was predominant at low sucrose concentrations, whereas above 25 mM a linear component following first order kinetics dominated. Sucrose uptake can be described by the the equation $v = V_{max}\, S/(S + K_m) + kS$, where v is the rate of sucrose uptake, S is the sucrose concentration in the incubation solution, V_{max} and K_m are Michaelis-Menten constants, and k is the first order rate coefficient (see Maynard and Lucas, 1982). Sucrose uptake kinetics showed a steeper linear component at high (k = 0.015 mmol g [fresh weight]$^{-1}$ h^{-1}) than at low (k = 0.011 mmol g [fresh weight]$^{-1}$ h^{-1}) turgor (Fig. 5-3A).

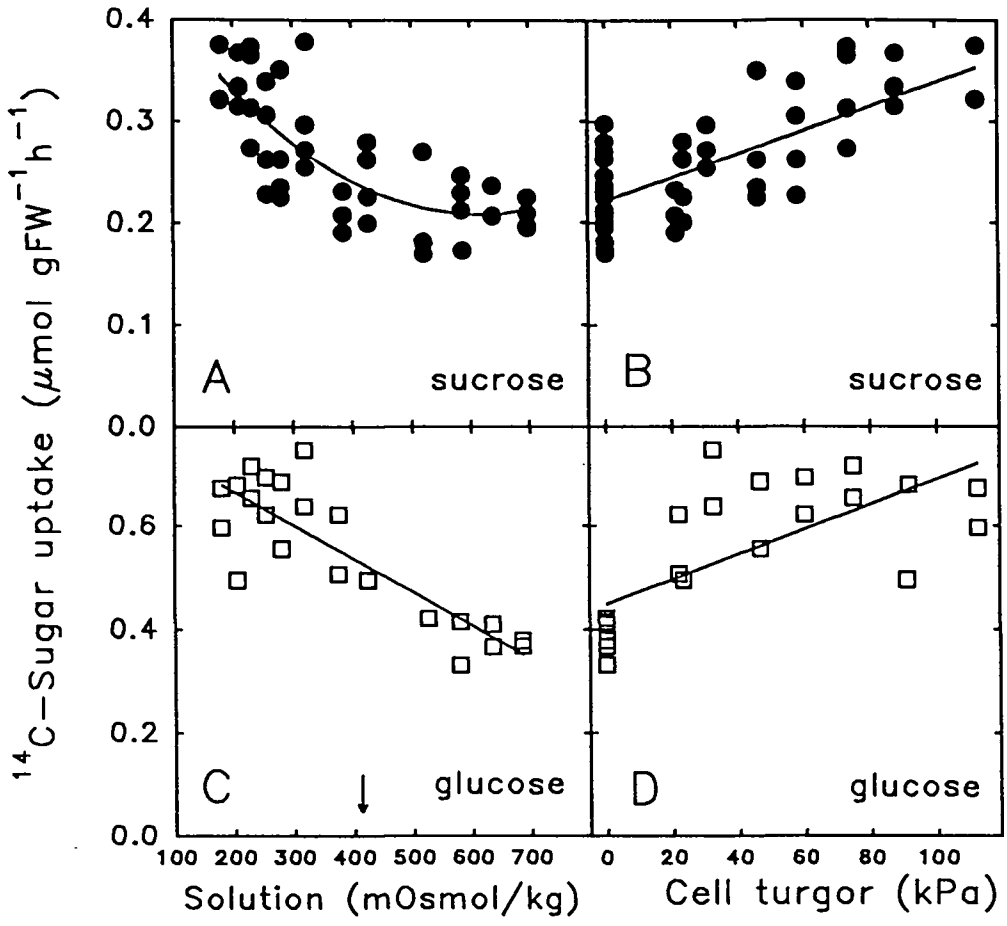

Figure 5-2. Uptake of [^{14}C]-sucrose (●) by strawberry fruit tissue disks (A) in incubation solutions at various osmolarities containing mannitol ($y = 0.4810 - 0.0010x - .0001x^2$; $r^2 = -0.61$) and (B) at the calculated cell turgor of the tissue ($y = 0.221 + 0.001x$, $r^2 = 0.59$). The arrow indicates the initial water potential of fruit tissue. Data presented are from four experiments, with each symbol the mean of ten disks or the incubation solution. Turgor was assumed to equal zero when solution osmolarity was > 450 mOsmol kg^{-1}. Uptake of [^{14}C]-glucose (□) by fruit tissue disks (C) in incubation solutions at various osmolarities containing mannitol ($y = 0.794 - 0.001x$, $r^2 = -0.74$) and (D) at the calculated cell turgor of the tissue ($y = 0.449 - 0.002x$, $r^2 = 0.49$). All regression coefficients were statistically significant from zero, $P<0.05$. Data presented are from two experiments, with each symbol the mean of ten disks or the incubation solution.

The higher turgor produced by the 265 mOsmol kg^{-1} solution also enhanced the saturable component of sucrose uptake, indicated by an increase in V_{max} (Table 5-1). The value of k , V_{max} and K_m for the uptake of sucrose at high turgor were similar to those previously reported by Forney and Breen (1986) who used buffer solutions that were not balanced osmotically (Table 5-1).

The saturable kinetics of glucose uptake were enhanced at the higher cell turgor (Fig. 5-3C, Table 5-1). In a separated experiment, O-methyl-glucose, a nonmetabolizable glucose analog, also exhibited enhanced saturable uptake at higher turgor (Fig. 5-3D). O-methyl-glucose saturable uptake curves were indistinguishable from glucose uptake curves when run simultaneously at respective turgors (data not shown).

[^{14}C]-mannitol, with sorbitol used as an osmoticum, was found to permeate the disk cells, but mannitol was still suitable as an osmoticum for altering disk cell turgor. Uptake of [^{14}C]-mannitol displayed only a linear uptake component that was steeper at high (k = 0.027 mmol [g fresh weight]$^{-1}$ h^{-1}) than at low (k = 0.024 mmol [g fresh weight]$^{-1}$ h^{-1}) turgor (Fig. 5-3B).

Addition of inhibitor - The saturable component of sucrose uptake was strongly inhibited by preincubation of disks in 2 mM PCMBS (Fig. 5-4A and 5-4B); this is shown by a 80% reduction in V_{max} and a quadrupling in the K_m value (Table 5-1). The linear component of sucrose uptake was again steeper at high than low turgor and was not inhibited at either turgor level by PCMBS (Fig. 5-4A, 5-4B; Table 5-1).

Sucrose efflux - The rate of efflux of ^{14}C from the cytoplasm and vacuole after disks were preloaded was not significantly affected by turgor, indicating that turgor had little effect

Table 5-1. Summary of kinetics constants for uptake of sugar in strawberry fruit disks. Sugar uptake can be described by the equation $v = V_{max} S/(S + K_m) + kS$, where v is the rate of sugar uptake, S is the sucrose concentration in the incubation solution, V_{max} and K_m are Michaelis-Menten constants, and k is the first order rate coefficient. Fruit tissue was at high turgor in incubation solutions at 265 mOsmol kg^{-1}, whereas tissue was at low or zero turgor in solutions at 455 mOsmol kg^{-1}.

Sugar	k (mmol $gFW^{-1}h^{-1}$)	K_m (mM)	V_{max} (mmol gFW^{-1} h^{-1})
Sucrose			
High turgor	0.015	2.3	0.242
Low turgor	0.011	3.1	0.164
Glucose			
High turgor	0.000	68.0	8.6
Low turgor	0.000	160.0	5.3
Sucrose			
High turgor	0.016	2.1	0.132
With PCMBS	0.015	5.3	0.029
Low turgor	0.012	1.0	0.118
With PCMBS	0.010	5.4	0.028

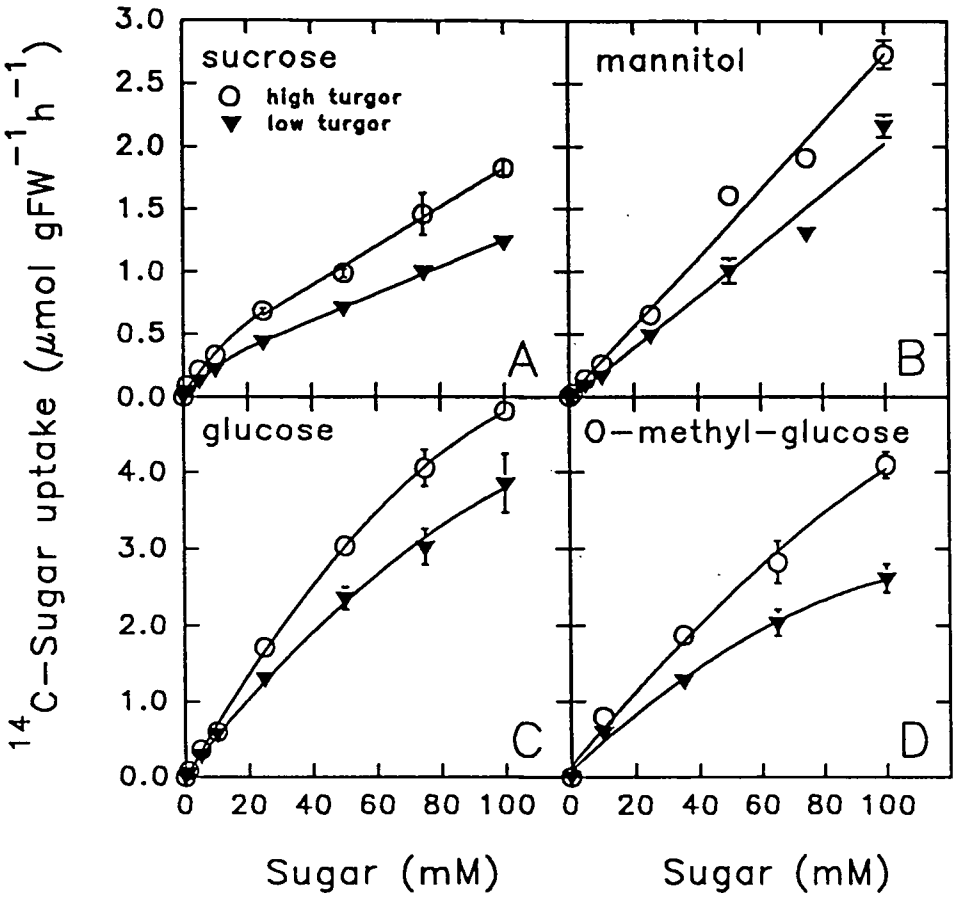

Figure 5-3. Concentration dependence of (A) [^{14}C]-sucrose, (B) [^{14}C]-mannitol, (C) [^{14}C]-glucose, and (D) O-methyl-[^{14}C]-glucose uptake by strawberry fruit disks at high (O) and low (▼) turgor. Incubation solutions were adjusted with mannitol so that the total osmolarity was maintained at all sugar concentrations. Symbols represent means of four replicates for [^{14}C]-sucrose and two replicates for all other sugars ± SE.

on the integrity of cell membranes or cell viability. There was a trend for low turgor disks to display shorter [^{14}C]-efflux half-life times. Figure 5-5 shows the time course reduction in the total ^{14}C remaining in fruit tissue disks at high or low turgor with continued rinsing used in determining [^{14}C]-efflux half-life times. The half-life for ^{14}C efflux from the vacuole was 407.0 min (±34.2 SE, n = 4) at high and 321.7 min (±29.6 SE, n=4) at low turgor (P-value > 0.2 using an independent t-test). For the cytoplasm, half-life for ^{14}C efflux was 18.5 (±3.9 SE, n = 4) at high turgor and 12.6 min (±5.5 SE, n = 4) at low turgor (P-value > 0.23 using an independent t-test).

Cell viability - Turgor effects were not the result of differential cell injury or death since estimates of cell viability in disks was unaffected by the osmolarity of buffer solutions. The efflux of phenolics from disks, a measure of cell injury, was similar (approximately 45% of total phenolics) at all solution osmolarities. Tissue viability was also assessed by measuring the percentage of cells in the outermost intact cell layer of disks which stained with Neutral Red. After 2 h incubation of ten disks in 150, 310 or 610 mOsmol kg^{-1} solutions, 39 to 48% of cells were stained. Regression analysis showed no difference between the staining of cells with increasing osmolarity. Only 16% of cells took up stain in disks incubated in distilled water for 2 h. Cells in disks that were frozen at -80°C or heated at 60°C for 1 h prior to incubation took up no stain.

5.5 Discussion

The effect of incubation solution osmolarity on uptake of sugars by tissue disks of strawberry fruit was due to differences in cell turgor, not cell osmotic potential. This was

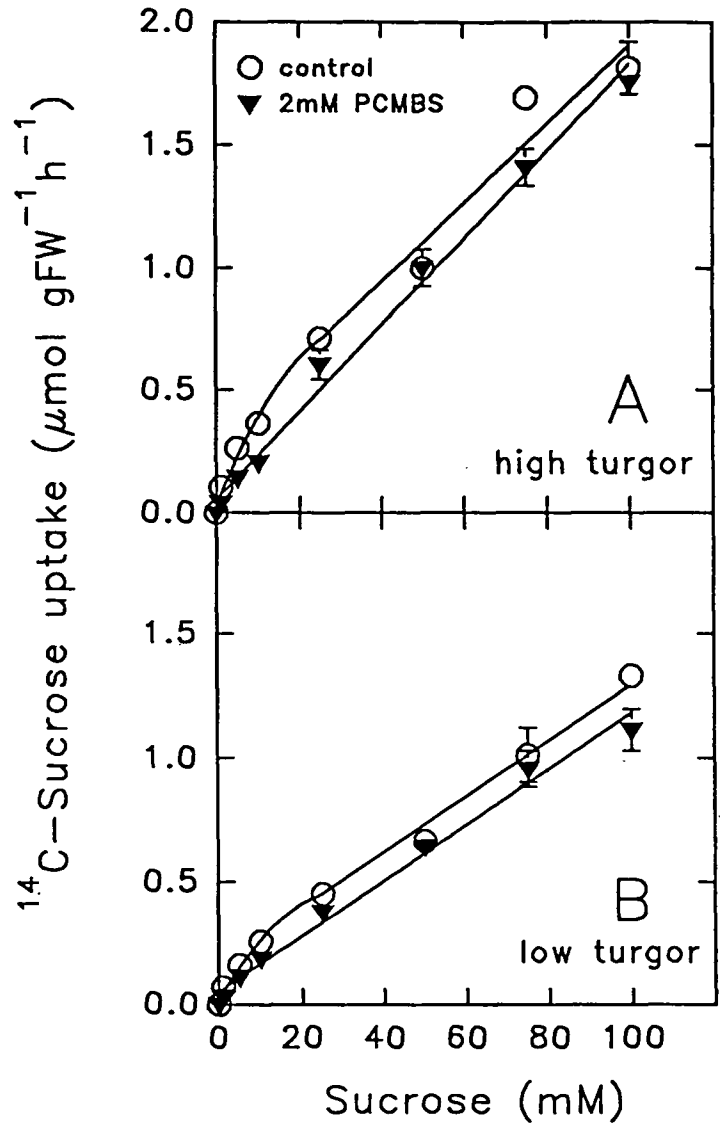

Figure 5-4. Concentration dependence of [^{14}C]-sucrose uptake by strawberry fruit disks, pretreatment with 2 mM PCMBS (▼) and without pretreatment (O) in mannitol containing incubation solutions at (A) high and (B) low turgor. Incubation solutions were adjusted so that the total osmolarity was maintained at all sucrose concentrations. Symbols represent means of two replicates ± SE.

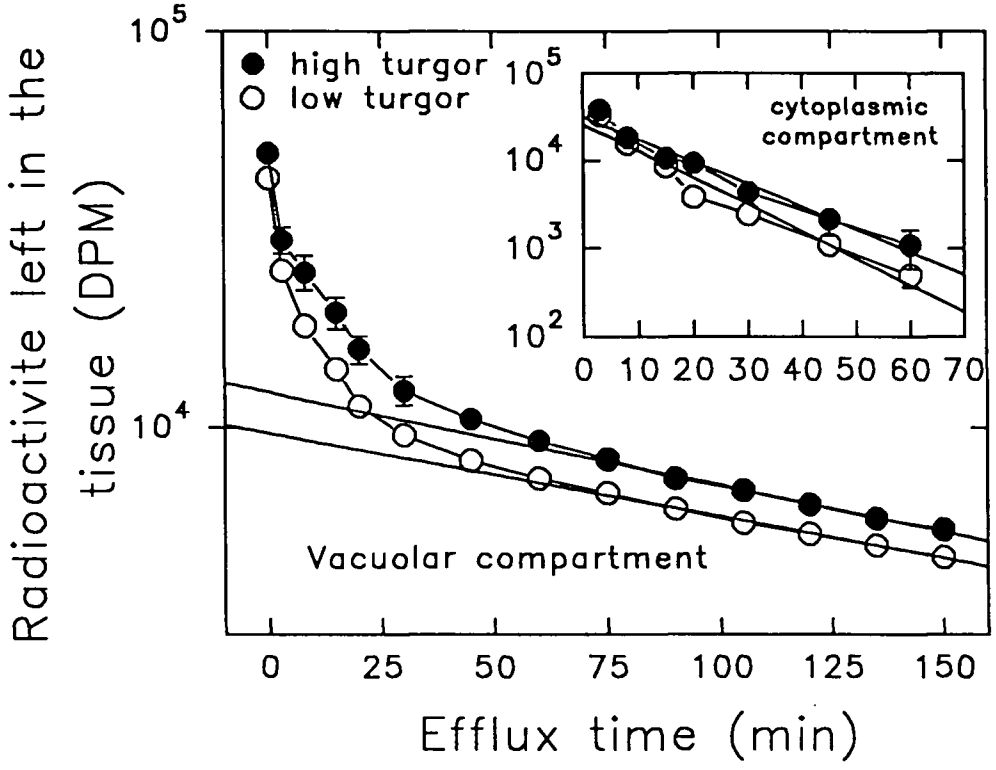

Figure 5-5. Time course reduction in the total ^{14}C remaining in fruit tissue disks at high (●) or low (O) turgor conditions with continued rinsing. Regression lines used to determine (A) vacuolar ^{14}C efflux and (B) cytoplasmic efflux, after subtraction of vacuolar contribution at high and low turgor. Symbols represent means of four replicates ± SE.

demonstrated by the response of uptake to the turgor-altering osmoticum, mannitol and the absence of a response with ethylene glycol, which modified cell osmotic potential but not cell turgor. Osmolarity of the incubation solutions did not affect cell injury or mortality.

The average turgor of the green-white strawberry fruit cortex (which is predominately parenchyma cells with some vascular tissue) prior to incubation was only 58 kPa, supporting the possibility of high apoplast solute levels. Lang and Thorpe (1986) have suggested that high concentrations of solutes in the apoplast of sink tissue reduce cell turgor and augment the phloem pressure gradient from the source to sink region. In potato tuber (sink) tissue, where sugar is thought to be unloaded from the phloem symplastically (Oparka and Prior, 1987), cell turgor has been calculated to be 320 kPa. In contrast, Shackel et al. (1991) used a pressure probe and found cell turgor in ripening tomato fruit to be less than 20 kPa; an apoplastic unloading pathway is thought to predominate at this developmental stage (Damon et al., 1988). Using *in situ* psychometric methodology, Pomper and Breen (1995, see chapter 4) calculated turgor of green-white and slightly pink strawberry fruit as 250 kPa and 50 kPa, respectively. They also found that solute levels in the apoplast increased as fruit developed from green-white to pink, as evidenced by apoplastic solute potentials decreasing from -0.25 and -0.41 MPa. The accumulation of solutes in the apoplast and the decrease in cell turgor in strawberry fruit after the green-white stage is consistent with, but not limited to, apoplastic phloem unloading. Maynard and Lucas (1982) pointed out that apoplastic uptake may represent a general retrieval system for sugars leaking from sink cells rather

than an integral component of apoplastic unloading. From studies on the uptake of [^{14}C]-sucrose and label redistribution of its hexose components, Forney and Breen (1986) suggested that both apoplastic and symplastic unloading are operating in strawberry fruit.

The uptake of both sucrose and glucose by strawberry fruit tissue decreased with a decrease in cell turgor (i.e. with an increase in osmolarity of the incubation solution). In contrast, tissues from potato tuber (Oparka and Wright, 1988a, b), sugar beet (Wyse et al., 1986), as well as *Phaseolus* leaves (Daie and Wyse, 1985) and young cotyledons (Patrick, 1994a, b) show an opposite response; a decline in turgor stimulates sugar uptake. To check our technique, we examined uptake of [^{14}C]-sucrose by (sink) potato tuber disks using our buffer solutions and sugar uptake protocol. We confirmed the results of Oparka and Wright (1988a, b) that uptake of [^{14}C]-sucrose increases with increasing osmolarity of mannitol incubation solutions (data not shown). Our observation of the opposite effect of reduced cell turgor on sugar uptake in green-white strawberry fruit disks suggests that a decline in cell turgor does not increase sugar accumulation in this tissue and the possibility that strawberry fruit cells at this stage are unable to osmoregulate.

Declining turgor also failed to enhance sucrose uptake in disks of *Citrus sinensis* leaves (Martinez-Cortina and Sanz, 1994) and carrot root tissue (Hole and Dearman, 1994). Hole and Dearman (1994) concluded that transmembrane exchange of sucrose is not involved in regulating solute potential in the apoplast of carrot root or in controlling turgor gradients between source and sink. However, these authors suggested that other osmotically active compounds in the sink apoplast, such as potassium (Enoch and Glinka, 1981), could still be involved in creating turgor gradients between source and sink.

Sucrose and hexoses in the apoplast of red ripe strawberry fruit make up only 35% of the total apoplastic solute potential of -1.0 MPa (Pomper and Breen, 1995, chapter 4). Therefore non-sugar solutes in the apoplast of strawberry fruit may play a more important role in regulating the apoplastic water potential (or turgor) gradient between the fruit and leaves.

In addition to the rate of uptake, turgor affected the kinetics of sugar uptake in strawberry fruit tissue in a manner different from that in sugar beet (Wyse et al., 1986), potato (Oparka and Wright, 1988b), and bean leaf disks (Daie and Wyse, 1985). Saturable (carrier mediated) uptake of glucose by strawberry fruit tissue was augmented at high turgor. The biphasic characteristic of sucrose uptake by fruit tissue was also more pronounced at high turgor, displaying an enhanced saturable component and an increased slope of the linear (diffusional) component. Conversely, higher cell turgor in sugar beet root (Wyse et al., 1986) and bean leaf (Daie and Wyse, 1985) disks reduced the saturable component of sucrose uptake by these tissues, but it did not affect the linear phase. Energy for carrier mediated (saturable) sucrose uptake is indirectly supplied via ATP (Bush, 1993) and inhibition of a plasma membrane H^+-ATPase by high turgor was suggested as the cause of the decline in the saturable component in sugar beet (Wyse et al., 1986). In tissue of young (sink) potato tubers, higher turgor decreased the slope of linear uptake, whereas the saturable component was unchanged (Oparka and Wright, 1988a, b). Inhibitor studies suggest that a turgor-sensitive H^+-ATPase is active in sugar importing (sink) tubers but not in sprouting (source) tubers (Wright and Oparka, 1989).

The saturable component of sucrose uptake in strawberry fruit was inhibited by PCMBS as has been demonstrated in many plant tissues (Giaquinta, 1976; Oparka and Wright, 1988a, b). The limited effect of PCMBS at sucrose concentrations greater than 25 mM suggests that carrier mediated uptake of sucrose contributes little to total uptake at high sucrose concentrations. PCMBS has been reported not to penetrate the plasma membrane (Giaquinta, 1976) and since sucrose uptake in untreated tissue was so similar overall to PCMBS-treated tissue, it seems likely that PCMBS did not penetrate into the cytoplasm where cellular metabolism and storage functions would have been significantly affected. Very little is known concerning the source of the linear (diffusional) component of sucrose uptake; it may represent a channel system, or even another carrier system not sensitive to PCMBS (see Bush, 1993; Lin, 1985). The uptake of [^{14}C]-sugars by the strawberry fruit tissue is a function of the combined effects of turgor on both plasma membrane and tonoplast transport arranged in series, so it is difficult to identify the regulatory influence of changes in cell turgor.

The kinetics of sugar uptake may reflect metabolism within cells in addition to transport across the plasma membrane. In sink potato tuber tissue, the biphasic kinetic pattern of glucose and fructose uptake represents a linear component of sugar transport across the plasma membrane superimposed on a second saturable component representing starch synthesis within cells (Wright and Oparka, 1990). In strawberry, only 20% of the ^{14}C of labeled sucrose is incorporated into an ethanol insoluble component representing starch and cell wall components during a 4 h uptake period (Forney and Breen, 1986). We confirmed this and found that changes in turgor did not alter the proportion of ^{14}C

incorporated into the insoluble component (data not shown). Increased uptake of the nonmetabolizable sugar O-methyl-glucose at high turgor also indicated that only carrier activity was influenced by cell turgor, not accumulation due to changes in cell metabolism.

Efflux rates from the cytoplasm and vacuole of strawberry fruit cells indicate that high turgor did not significantly increase leakage of ^{14}C from cell membranes. Half-life times for [^{14}C]-efflux from vacuoles of sugar beet taproot tissue disks were shorter at high (522 min) than at low (1363 min) cell turgor (Wyse et al., 1986) whereas comparable values for strawberry fruit tissue were similar, 407 and 321 min respectively. The half-life times for the efflux of ^{14}C from the cytoplasm were similar in sugar beet taproot (Wyse et al., 1986) and strawberry fruit tissue at high or low turgor. The shorter vacuole efflux times in strawberry fruit tissue, as compared to those of sugar beet taproot tissue, suggests that the fruit tissue is more prone to leakage of sugars stored in the vacuole. Retrieval of sugars that leak into the apoplast may be a higher priority in strawberry fruit tissue than in the less permeable sugar beet taproot tissue.

Cram (1984) has pointed out that mannitol may be suitable as an osmoticum in incubation solutions only in short term experiments of several hours, since in some species or tissues this sugar alcohol is taken up and utilized, possibly affecting the metabolism of the cell. Mannitol was found to permeate strawberry cells in this study, but was still suitable as an osmoticum for altering disk cell turgor. Since mannitol uptake increased at higher turgor and a saturable carrier uptake was not present, this can be taken as evidence of increase membrane permeability with increased turgor. However, not only

does the [^{14}C]-efflux data at high turgor not support this, we also found that shocking strawberry tissue disks in dilute buffer solutions and then placing them at low turgor conditions did not significantly increase [^{14}C]-sucrose uptake compared to that of unshocked tissue, indicating that high turgor conditions did not cause significant membrane damage (data not shown).

In conclusion, cells of strawberry fruit at the green-white stage do not possess sucrose and glucose uptake systems that are stimulated by a reduction in turgor nor does turgor significantly modify sucrose efflux. If the results of the *in vitro* sugar transport experiments of the present study reflect *in vivo* transport mechanism, it would seem unlikely that strawberry fruit cells are able to respond to, or regulate, the levels of apoplastic solutes through sugar transport. However, since sucrose and hexoses account for only about 35% of the osmotic potential of the apoplastic solution in strawberry fruit (Pomper and Breen, 1995, chapter 4), the cellular response to apoplastic solutes could be mediated by non-sugar components.

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CHAPTER 6

EPILOGUE

An improved understanding of the mechanisms of water and sugar transport in strawberry could help identify strategies to increase transport to the fruit and influence its expansion. The objectives of this thesis research were to: 1) relate plant water relations to fruit expansion during water-stress, 2) to assess the contribution of apoplastic solutes toward fruit ψ_{aw}, and 3) determine whether the uptake of sugars by fruit cells form the apoplast was turgor-sensitive. The major conclusions of this research were that: 1) green-white fruit expansion was very sensitive to water stress, occurring when leaf ψ_w was higher than that of fruit, and that fruit underwent osmotic adjustment when plants were subjected to water-stress, but not to levels that allowed continued expansion, 2) apoplastic solutes (mainly non-sugar components) contributed significantly to the fruit ψ_{aw} late in development, and 3) sugar uptake from the apoplast by fruit cells was not stimulated by reductions in turgor as in tissues of many other plants. In this epilogue, I will comment on some of the methods used in this research and explore some of the potential avenues to further our understanding of water and sugar transport to the developing strawberry fruit.

Is the ψ_w of plant tissues and organs meaningful as an indicator of water status or predictor of water transport? After several decades of emphasis on plant ψ_w as a good indicator of plant water status, some investigators claim that physiological processes are

better related to RWC than to ψ_w (Sinclair and Ludlow, 1985). Rates of cell enlargement, photosynthesis, and respiration are highly correlated with RWC (Kramer and Boyer, 1995). The main advantage of using ψ_w is that it is based on a physically defined reference rather than a biological one (e.g. pure free water at atmospheric pressure and a defined gravitational position, at the same temperature as the system of interest), allowing an experiment to be repeated and compared to a defined reference (Boyer, 1988). Comparisons of plant water status based on water content are based on a biological reference that varies, although RWC is an improvement because it uses the water content of cells at full turgor as a reference. There is also the question of whether a growing organ or tissue, such as a green-white strawberry fruit can attain full turgor. The ψ_w, as well as RWC, both provide useful information on tissue water status under stress, but ψ_w may be more reliably measured than RWC in growing tissue.

There is continuing evidence to reject the belief that water movement within a plant can be predicted solely on differences in ψ_w (Passioura, 1988). However, in this present study, green-white strawberry fruit expansion (indicating water flow to the fruit) did occur when leaf ψ_w was higher than that of fruit, as would be predicted by the difference in ψ_w. A direct measure of water movement through the xylem to the fruit would still be more desirable. Methods that would seem to allow the examination of xylem water movement in the plant that at first seem attractive, such as dye movement in the apoplast, often do not represent the flow of water because of barriers in the apoplast restricting movement of dye molecules but not to water (see Canny, 1993). Nuclear resonce imaging (NRI) has been used to examine the amount of bound and free water in

plant tissues, including the vascular system. Maas and Line (1995) have completed studies of developing strawberry fruit using this technique and identified higher levels of free water in vascular traces as compared to parenchyma tissue. The NRI apparatus is very expansive and requires excellent technical expertise. However, it seems possible that NRI could be used in examining changes in water status of fruit with water-stress and diurnal changes in water transport.

What proportion of water transported into developing strawberry fruit is delivered by the phloem? The contribution of phloem delivered water to developing strawberry fruit could be determined by girdling the pedicel and examining the change in the rate of expansion. 'Brighton' strawberry fruit offer a unique opportunity to examine the influence of cell turgor on phloem unloading of assimilate into the fruit. Fruit of this cultivar form a large hollow cavity as it ripens. This cavity could be filled with solutions of non-permeating osmotica in an effort to alter fruit turgor and influence phloem unloading of radioactively labeled sucrose into the cavity and fruit tissues.

The active accumulation of compatible solutes in plant tissue with osmotic adjustment, such as sugars and amino acids, is generally thought to be a mechanism by which positive turgor is maintained in cells during water-stress. However, Passioura (1988b) has argued that accumulation of these solutes, such as glucose and sucrose, could also just be a by-product of the slow down in use of solutes by tissues during water-stress and this view should be considered when interpreting osmotic adjustment studies. The observed relative increase in glucose compared to sucrose in water-stressed green-white strawberry fruit tissue suggests that part of the osmotic adjustment mechanism in stressed

fruit could be hydrolysis of stored sucrose by either invertase or sucrose synthase or combination of the two. It would be interesting to examine the activities of these two enzymes in control and water-stressed strawberry fruit. Tarczynski et al. (1993) increased the salt tolerance of tobacco plants by introducing a bacterial gene that increased the production of mannitol in the plant tissues. Could increased water-stress tolerance be introduced into strawberry without disturbing normal metabolic pathways? Sugar uptake from the apoplast of green-white fruit tissue was not stimulated by reduced cell turgor, suggesting this process is not involved in osmotic adjustment in fruit tissues. Does reduced turgor in fruit cells increase apoplastic uptake of solutes other than sugars or does it increase phloem transport of solute to the fruit leading to a decrease in fruit ψ_s? There are many questions that remain to be answered.

Let's step back for a minute and look at the whole plant. Longer-term adaption of the strawberry plant to drought was not examined in this study. In addition to the occurrence of leaf osmotic adjustment, Save et al. (1993) reported morphological adaptions with long-term drought stress of the cultivated strawberry in the field. They found that drought stress reduced total leaf area and altered the plant architecture into a polylayer canopy with leaves oriented more toward the north. Drought stress did not increase root biomass (g dry weight plant^{-1}). Gains in drought resistance resulting in increase fruit yield in strawberry will likely require consideration of the mechanisms plant adaption at the whole-plant and cellular levels.

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APPENDICES

APPENDIX A

IDENTIFICATION OF BOTH ACID AND ALKALINE INVERTASE ACTIVITY IN DEVELOPING STRAWBERRY FRUIT

A.1 Abstract

Invertase may influence sugar levels and assimilate transport in strawberry fruit. Several groups, including our own, only detected acid invertase activity (optimum pH 4.6) in strawberry fruit, however, recently Hubbard et al. (Physiol. Plant. 82:191-196, 1991) reported the presence of alkaline invertase activity (pH 7.5). Since dissimilar isolation protocols and cultivars may have contributed to the different findings, we re-examined our work with developing 'Brighton' strawberry fruit using the extraction procedure of Hubbard et al. Alkaline invertase was detected and its activity increased many fold as fruit developed from green to the red stage, whereas, acid invertase activity decreased markedly. In addition, when fruit extracts were precipitated with cold acetone, a precipitate contained 60% of the acid invertase activity and the protein coagulated at the surface contained 60% of the alkaline invertase activity. This allowed easy separation of these two enzymes.

A.2 Introduction

Sugar concentrations of strawberry fruit have been widely studied as a component of fruit flavor (Shaw, 1988). This laboratory continues to be interested in the

involvement of invertase (which degrades sucrose to hexoses) in sucrose transport and sugar accumulation in strawberry fruit. Poovaiah and Veluthambi (1985) reported the presence of only acid invertase activity (optimum activity at pH 4.6) in the soluble and cell wall extracts of developing 'Ozark Beauty' strawberry fruit. Soluble acid invertase activity was highest at anthesis after which it receded rapidly with fruit development. Ranwala et al. (1992) also reported the presence of soluble acid invertase in 'Hokowase' strawberry fruit, however, the activity (on a fresh weight basis) was higher in ripe than unripe fruit. Recently, Hubbard et al. (1991) reported the presence of both soluble acid and alkaline invertase in 'Chandler' strawberry fruit, with highest acid activity in unripe fruit and maximum alkaline activity in ripening fruit.

In our laboratory, using the extraction protocol of Poovaiah and Veluthambi (1985), Scott (1989) reported that 'Brighton' fruit contained only soluble acid invertase activity (pH 4.6) with highest early in fruit development. Different cultivars were used in the above studies and we wished to determine if this, or different extraction protocols influenced the ability to detect alkaline invertase activity. Therefore we re-examined invertase activity in developing 'Brighton' strawberry fruit using the protocol of Hubbard et al. to determine if alkaline invertase was present.

A.3 Materials and methods

Plant material - Plants of 'Brighton', a day-neutral strawberry were grown in 3.8-liter pots in 1 soil: 1 coarse sand: 1 peat (by volume), in a greenhouse that was maintained at 30/18°C (day/night), and where high-pressure sodium lamps (175 ±25 mmol m^{-2} s^{-1})

provided a 14-h photoperiod. Primary flowers were pollinated with a small paint brush, tagged, and all competing flowers or flower buds removed from an inflorescence. Fruit were harvested at small green, green-white, and red-ripe stages of development, representing 7, 15, and 25 days after anthesis (DAA).

Extraction protocol - Protein extraction of alkaline and acid invertase were performed as described by Hubbard et al. (1991). Briefly, fruit samples were powdered in a chilled mortar and pestle with liquid nitrogen. The fruit powder was then combined with a homogenizing buffer (8 ml/gFW) containing 100 mM 3(N-morpholino) propanesulfonic acid (MOPS) (pH 7.5), 5 mM $MgCl_2$, 0.5 mg ml^{-1} bovine serum albumin (BSA), 2.5 mM dithiothreitol (DTT), 0.05% (v/v) Triton x-100, 10 mM ascorbic acid, 2% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% (W/V) polyvinylpyrrolidone. Tissue homoginates were centrifuged for 30 sec at 10,000xg, and the supernate desalted by centrifugal column filtration (Helmerhorst and Stokes, 1980). All operations were conducted at 4°C.

In separate experiments, tissue homoginates from green-white fruit (15 DAA) were desalted by precipitating the protein overnight at -20°C using 5 volumes of cold cetone:1 per volume of fruit extract instead of the centrifugal column filtration method used by above.

Acid and alkaline invertase activity assays - Reaction mixtures (100 ml) containing buffers at 100 mM citrate-phosphate (pH 3.5-5.0), 2(N-morpholino) ethanesulfonic acid (MES, pH 6.0), MOPS (pH 7.5), and N-2-hydroxyethylpiperazine N'-2-ethanesulfoic acid (HEPES, pH 8.0-8.2), with 120 mM sucrose and desalted extract (40 ml) were used to

determine the pH optimum of invertase activity in the fruit extract. Reaction mixtures were terminated by placing tubes in boiling water at 0 and 30 min after initiation with enzyme extract. Reaction mixtures were assayed for glucose using the glucose oxidase, peroxidase and o-dianisidine system from Sigma Chemical, St.Louis, Mo. (Sigma Technical Bulletin No. 510).

Protein precipitates or flocculent from cold acetone were, centrifuged for 30 min at 4°C at 3000xg, decanted, and the protein re-suspended on ice with 1 ml of buffer (10 mM MOPS at pH 7.5), and 40 ml of extract assayed for invertase activity as above.

A.4 Results and discussion

Using the protein extraction method of Hubbard et al. (1991) we were able to recover both acid (pH 4.0) and alkaline (pH 8.0) invertase activities from 'Brighton' strawberry fruit. Essentially only acid invertase activity was isolated from small green fruit, activity was highest at pH 4.0 and declined steadily as pH increased (Fig. A1). Both acid and alkaline invertase activity were isolated from green-white fruit, with alkaline activity highest at pH 8.0. In red fruit, high alkaline invertase activity was isolated and acid invertase activity was reduced from earlier developmental stages.

When the maximum activities (g fresh weight^{-1}) from Figure A1 are compared in Table A1, acid invertase activity (pH 4.0) decreased by nearly 50% from the green-white to red fruit stage. Alkaline invertase activity (pH 8.0) was extremely low in small green fruit, but increased several fold with further fruit development.

The high acid invertase activity in small green fruit of 'Brighton' and its decline with further development is consistent with earlier findings (Scott, 1989). The rise in alkaline invertase activity in this cultivar with fruit development is similar to that reported by Hubbard et al. (1991) in 'Chandler' fruit. Although the role of alkaline invertase is unclear, the rise in its activity during strawberry berry fruit ripening presumably influences the levels to which sucrose and hexose accumulate in ripe fruit.

The previous failure of Scott (1989) to recover alkaline invertase activity from 'Brighton' fruit presumably was due to the use of the isolation protocol of Poovaiah and Veluthambi (1985). This protocol differs in many ways from the method of Hubbard et al. (1991), and therefore it is difficult to identify those aspects which allowed the extraction of alkaline invertase activity. However, in the methodology of Poovaiah and Veluthambi (1985), no protease inhibitors or BSA were included in the extraction buffer and that extract was dialyzed overnight. In contrast, the protocol of Hubbard et al. (1991) uses a large quantity of BSA in the extraction buffer and this may protect invertase from protease activity. Additionally, fruit homoginates are desalted immediately after extraction and invertase activity measured. Ranwala et al. (1992), with a protocol similar to Poovaiah and Veluthambi, also did not identify alkaline invertase activity and they also dialyzed fruit extracts overnight.

In a separate series of experiments, acid and alkaline invertase activities were found to separate when precipitated with cold acetone (Table A2). A protein flocculent formed in the acetone and upon centrifugation the resulting pellet contained 66% of the acid invertase activity, whereas protein that coagulated at the surface, when decanted and

centrifuged into a pellet, contained 61% of the alkaline activity. Lipids and proteins with associated lipids, would be expected to accumulate at the top of the acetone layer during protein precipitation. The accumulation of alkaline invertase activity in this region may be an indication that this enzyme is a membrane-bound protein in strawberry fruit. However, the literature does not contain any support for or against alkaline invertase being membrane bound. This method of separating the two invertase activities in the fruit would be useful in further efforts in characterizing of the enzymes.

In an attempt to further separate the acid and alkaline invertase activities, protein samples obtained through acetone precipitation were separated using non-denaturing polyacrylamide gel electrophoresis (PAGE) or native PAGE and gels stained for invertase activity (25 or 50 mg per lane) (von Schaewen et al., 1990). Unfortunately, we were unable to identify invertase activity in the strawberry fruit protein extracts on native gels assayed at pH 5.0 or 8.0, but yeast invertase used as a control was well stained (data not shown). The large amount of BSA added during the extraction may not have allowed enough fruit protein (including invertase) to be loaded onto the gels to allow detection of invertase activity. Based on this work, fruit protein extract obtained using the protocol of Hubbard et al. is not suitable for use with native PAGE methodologies as a method to further characterize strawberry invertase activity.

Hubbard et al. (1991) found that activities of the sucrose degrading enzymes, sucrose synthase, and alkaline invertase increased in activity along with a rise in sucrose content during ripening of strawberry fruit. The activity of the sucrose synthesizing enzyme, sucrose phosphate synthase, also increases during ripening. Increasing activities

sucrose synthesizing and degrading enzymes suggest that sucrose turnover occurs in strawberry fruit (Hubbard et al., 1991). It is interesting, that Ranwala et al. (1992) reported acid invertase activity was higher in ripe than unripe strawberry fruit, while Hubbard et al. (1991) reported the opposite. These groups used different cultivars, suggesting that there is variation in invertase activity amongst strawberry genotypes. Although Poovaiah and Veluthambi (1985) reported the presence of cell wall bound acid invertase activity in strawberry fruit, we did not measure its activity in the course of this present study.

Shaw (1988) found a highly positive genotypic correlation (1.00) between glucose and fructose in fruit, and a highly negative genotypic correlation (-0.66) between sucrose and glucose-fructose in a specific strawberry population. Sucrose, is the major sugar translocated in strawberry (Forney and Breen, 1985) and based on ^{14}C-sugar uptake, sucrose is hydrolyzed and re-synthesized upon uptake by strawberry fruit (Forney and Breen, 1986). Shaw (1988) suggests that glucose and fructose content in strawberry fruit is dependent on hydrolysis of translocated sucrose and that a single gene controls the level of sucrose in the fruit. This gene could very well be an absence of the sucrose synthesizing enzyme such as sucrose phosphate synthase, or high levels of alkaline or acid invertase activity in ripening fruit. Examination the germplasm of Shaw (1988) with the protocol of Hubbard et al. (1991) could identify whether high alkaline invertase activity in ripening strawberry influences sucrose levels in the fruit.

In conclusion, alkaline invertase activity was essentially absent in young green 'Brighton' fruit, but relatively high in green-white and red fruit. Alkaline and acid

invertase activities were partially separated when fruit protein was precipitated with cold acetone.

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Table A1. Acid and alkaline invertase activities during fruit development in 'Brighton' strawberry.

	Invertase activity (mmol $gFW^{-1}h^{-1}$)	
Fruit stage Days after anthesis(DAA)	pH 4.0	pH 8.0
Small green (7 DAA)	11.6 ±2.5 a*	0.2 ±0.1 a
Green-white (15 DAA)	7.8 ±1.4 a	6.4 ±1.7 b
Red (25 DAA)	4.2 ±1.0 b	9.2 ±1.3 c

*Separation of means by Duncans separation of means at a level of significance of 5%, means of 3 separate experiments with 3 replicates each, ±S.E.

Table A2. Acid and neutral-alkaline invertase activities separate during acetone precipitation.

	Invertase activity (mmol glucose $gFW^{-1}h^{-1}$)	
Protein acetone position	pH 4.0	pH 8.0
Top	2.8 ±0.1*	4.2 ±0.4
Bottom	5.5 ±0.6	2.7 ±0.2

* Note: mean of 2 separate experiments with 3 replicates each; ±S.E.

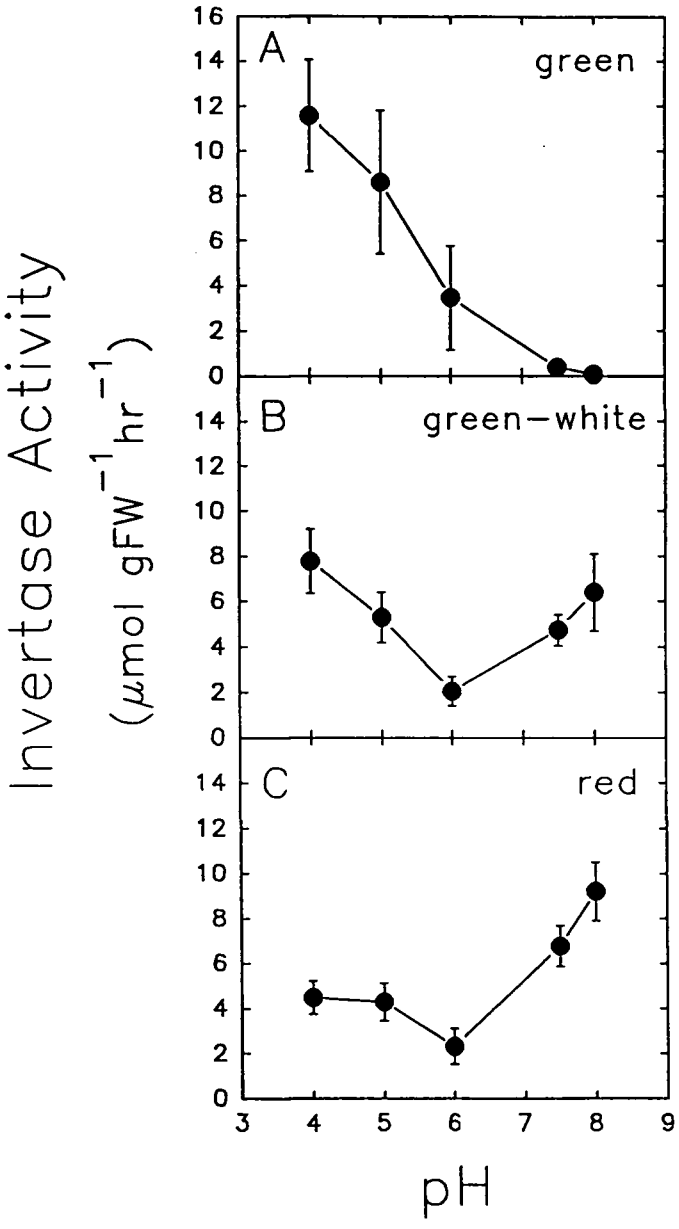

Figure A1. The activity-pH response of soluble invertase at three stages of strawberry fruit development. (A) Small green fruit showed only acid invertase activity (highest at pH 4.0). (B) Green-white fruit showed both alkaline invertase activity (highest at pH 8.0) and acid invertase activity. (C) Red fruit showed alkaline invertase activity and low acid invertase activity (pH 4.0).

APPENDIX B

FRUIT WATER LOSS AND ITS INFLUENCE ON WATER POTENTIAL

B.1 Introduction

Gradients in apoplastic water potential (ψ_{aw}) between plant organs due to differences in water loss, solute content and cell expansion can influence the transport of water and assimilate. The objectives of this study were to determine: 1) the rate of water loss from developing strawberry fruit, 2) if restricting fruit or leaf transpiration increased fruit ψ_{aw} while attached to the plant, and 3) relationship of the rate of change of fruit ψ_{aw} loss of water from a fruit being severed from the plant.

B.2 Materials and methods

1) Loss of water from attached strawberry fruit was estimated by measuring the decrease of fresh weight of detached fruit. Solitary primary fruit at small green, green-white, and red stages were harvested in the mid-afternoon by cutting the pedicel at a length of about 2 cm, the cut end was sealed with silicon grease, and the fruit placed on a greenhouse bench, out of direct sun light. Fruit were weighed at 0, 3, and 24 h after picking.

2) ψ_{aw} of green-white and red fruit and leaves was measured via *in situ* psychrometry in the greenhouse as described in Chapter 3). Weight loss and ψ_{aw} of red and green-white fruit were monitored over 10 h after fruit were severed from plants.

B.3 Results

1) Red fruit were about 10x and green-white fruit 6x larger than in terms of fresh weight and small green fruit (Table B1). Weight loss per unit fruit fresh weight was about 2x higher for small green fruit than fruit ant the other stages. Webb (1973) had reported that red fruit lost weight faster than immature fruit, depriving developing fruit within the inflorescence. However, the immature fruit used in that study were larger that the small green fruit used in this present study. This work suggest small 'Brighton' fruit lose water at a faster rate (g fresh weight $^{-1}$) than red fruit.

2) Reducing the transpiration of a green fruit by enclosing it in a clear plastic bag in mid-afternoon did not influence its ψ_{aw} (Fig. B1). The ψ_{aw} of the bagged fruit was similar to that of a comparable fruit in untreated plants. Bagging the entire plant increased leaf ψ_{aw}, but did not change the ψ_{aw} of green fruit. Bagging red fruit also did not affect fruit ψ_{aw} (Fig. B2). Again enclosing the entire plant increased leaf ψ_{aw}, while not changing red-fruit ψ_{aw}.

3) Upon severing fruit from the plant, the mean rate of weight loss per unit fresh weight was similar in green-white and red fruit, with fruit losing approximately 5% of their initial fresh weight after 12 h (Fig. B2). Severed green-white fruit showed a steady decline in ψ_{aw} (-1.00 MPa after 10 h), whereas ψ_{aw} of severed red fruit dropped much less (-0.35 MPa).

B.4 Conclusions

1) Small green strawberry fruit of the cultivar Brighton lose water at a faster rate (g fresh weight^{-1}) than red fruit.

2) Restricting water loss of fruit attached to the plant did not influence its ψ_{aw}.

3) Maintenance of ψ_{aw} of green-white fruit depended on constant delivery of water from the plant. However, ψ_{aw} red fruit was little influenced by removal from pedicel delivery of water.

B.5 Literature cited

Webb, R.A. 1973. A possible influence of pedicle dimensions on fruit size and yield in strawberry. Sci. Hort. 1:321-330.

Table B1. Water loss from 'Brighton' strawberry fruit in terms of weight loss after harvesting[z].

Stage	Initial fresh weight (g)	Loss of weight in mg g fruit^{-1} h^{-1} 0-3 h	0-24 h
Red	13.32 a[y]	9.7 a	5.5 a
Green-white	6.86 b	10.6 a	5.8 a
Small green	0.98 c	26.7 b	13.1 b

[z]Data is the mean of 10 fruit.
[y]Mean separation within columns by Duncan's multiple range test, P = 0.05.

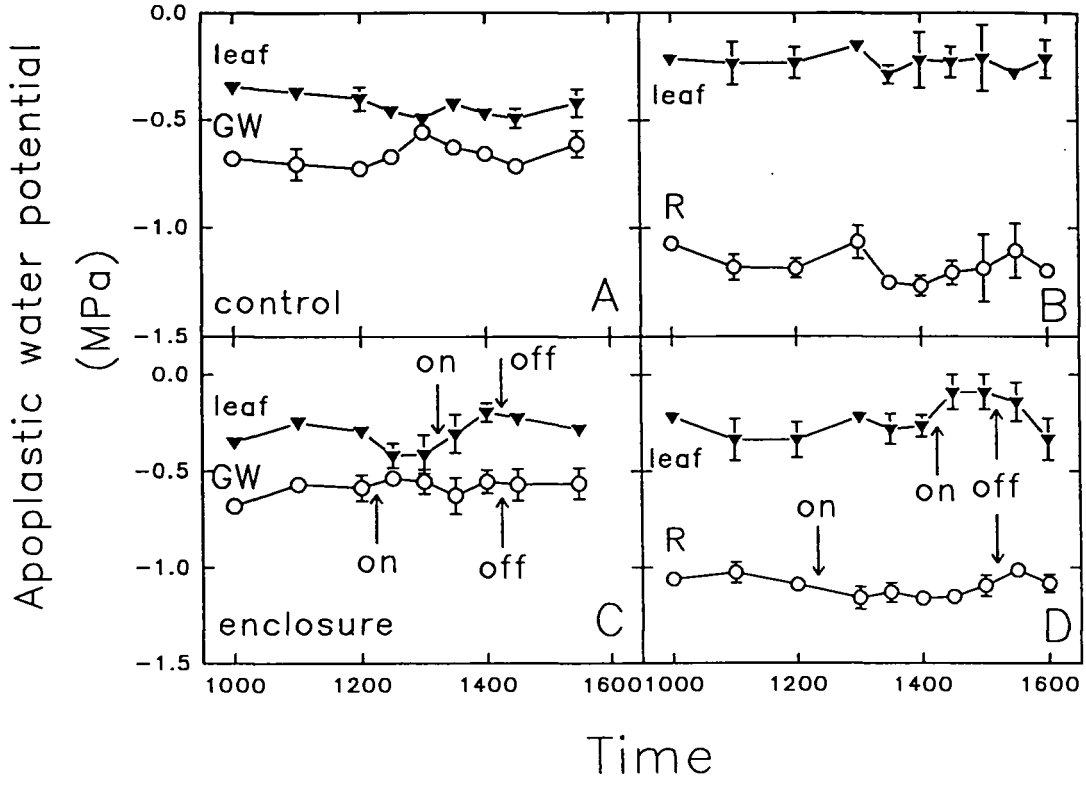

Figure B1. Restriction of transpiration of fruit and leaves. The apoplastic water potential (ψ_{aw}) of (A) control green-white (GW) fruit and leaves and (C) treated plant, and (B) control red (R) fruit and leaves and (D) treated plant. Arrows on treated plants indicate where plastic bags with moist paper towels were place either over the fruit or plant and then removed. ±SE. n = 3.

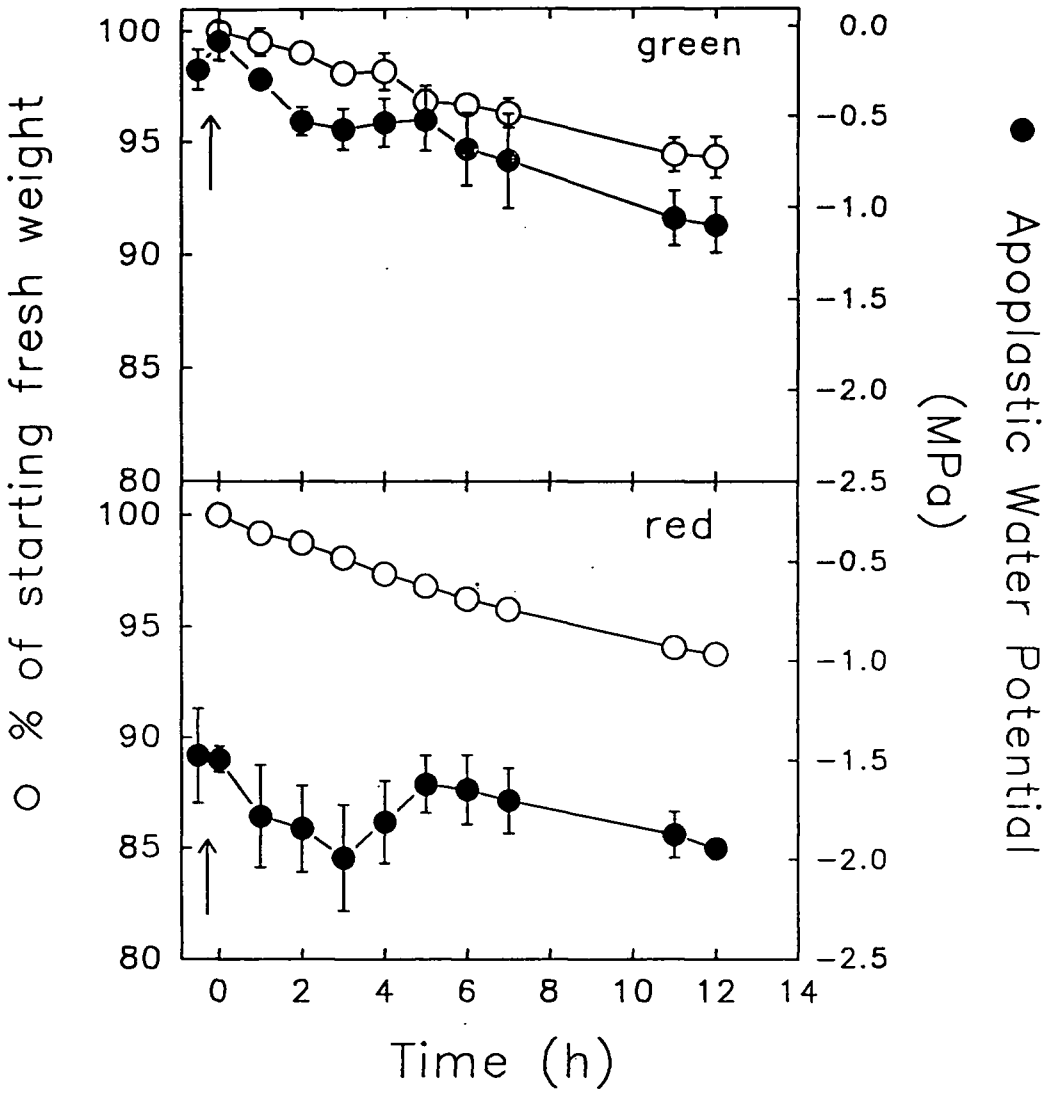

Figure B2. The apoplastic water potential (ψ_{aw}) of green-white (A) and red (B) 'Brighton' strawberry fruit. Arrow marks where fruit were severed from the plant. ±SE. n = 3.

APPENDIX C

THE BASIS OF THERMOCOUPLE PSYCHROMETRY AND HYGROMETRY

The water potential of a plant tissue can be determined by measuring the vapor pressure of the air that is in equilibrium with the tissue in a chamber at a known temperature, conveniently measured with a thermocouple. A thermocouple operates on the principle of the thermoelectric effect: such that when a circuit consisting of two wires made of different metals (e.g. copper and the alloy constantan) are joined at two junctions at different temperatures, a current flows around the circuit. When one junction is at a known temperature and the current is measured, the temperature of the other junction can be calculated. In psychrometry, organs can be severed, placed in an enclosed chamber and the water vapor of the air allowed to reach an equilibrium with the tissue. Inside the psychrometer chamber is a thermocouple whose junction is cooled to a temperature below the dew point of the chamber by the passage of an electrical current thus causing water to condense on the junction. (This cooling phenomenon, which is the opposite of the thermoelectric effect, is called the Peltier Effect: when a current is passed through a circuit of the two different metals one will heat and one will cool depending on the direction of the current.) The cooling current is then discontinued, and the condensed water allowed to evaporate from the junction into the surrounding atmosphere. The evaporating water draws heat from the junction (the heat of vaporization), depressing the temperature of the junction from that of the surrounding air temperature. The magnitude

of the temperature depression depends on the humidity of the surrounding air; the drier (and warmer) the air, the faster the evaporation rate and the greater the depression. The temperature of the junction is measured by electrical means prior to the cooling and during evaporation. The differential temperature of the junction is directly related to the relative humidity and hence the water potential measured (Wescor HR33T Dew Point Microvolt instruction and service manual). The ψ_w of a plant tissue can be determined with the equation: ψ_w (in MPa) = -1.06 T $\log_{10}$(100/RH) where T is the temperature (in Kelvins) and RH is the relative humidity (Salisbury and Ross, 1992).

The dewpoint or hygrometric method can theoretically yield more accurate estimate of ψ_w than the psychometric method. With psychrometry measurements, the wet junction will always be below the temperature of its surroundings and heat will also tend to flow from the surroundings to the junction. After an initial cooling of the junction, a dewpoint mode uses Peltier cooling to counter the inflow of heat electronically through pulses of current for cooling that exactly balance the heat inflow for a net energy transfer of zero. When this balance is set up on a dry thermocouple to account for all heat transfer mechanisms other than condensing or evaporation water, the temperature of a wet junction will be influenced only by the water and not the surrounding environment. This method requires that specific cooling coefficients be determined in advance of ψ_w measurements for each thermocouple for all temperatures at which it is used. Psychrometers (using the psychometric mode) or hygrometers (using the dewpoint mode) can be attached *in situ* on a plant so that the ψ_w of a plant organ can be used continuously to measure under various conditions over time. The same thermocouple apparatus can be

used with either the psychometric or dew point mode, the choice of mode is dependant on whether the microvoltmeter used for measurements contains the necessary electronics for the dew point procedure.

Literature cited

Salisbury, F.B. and C.W. Ross. 1992. Plant Physiology, 4th ed. Wadsworth Publishing Company, Inc. Belmont, CA.

APPENDIX D

ADDITIONAL GROWTH INFORMATION FOR SOLITARY 'BRIGHTON' FRUIT

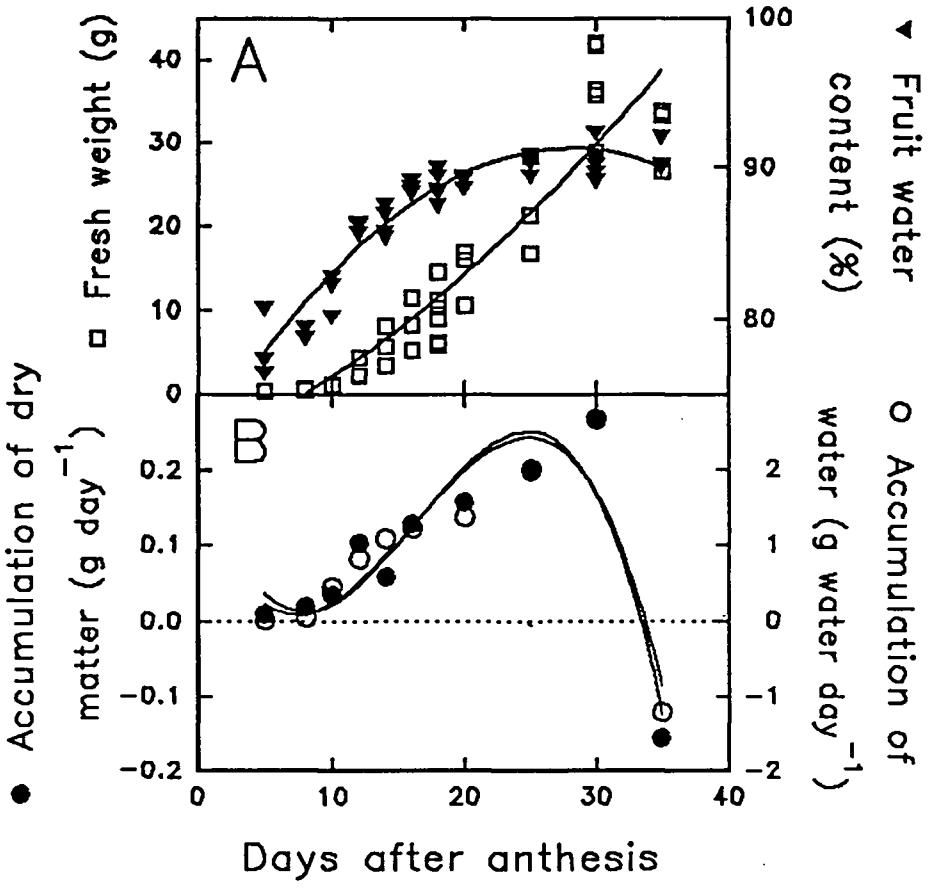

Figure D1. Growth data for developing solitary 'Brighton' fruit where competing flowers were removed at pollination. (A) fresh weight (; $y = -6.8 + 0.7x + 0.02x^2$, $r^2 = 0.87$, n = 40) and water content (∇; $y = 71.25 + 1.43x - 0.03x^2$, $r^2 = 0.91$) during fruit development. (B) Absolute growth rates in terms of dry matter accumulation (O; $y = 0.2170 - 0.0580x + 0.0050x^2 = 0.0009x^3$, $r^2 = 0.83$) and accumulation water (●; $y = 1.2903 - 0.3893x + 0.0359x^2 - 0.0007x^3$, $r^2 = 0.83$) during fruit development.

APPENDIX E

SCANNING ELECTRON MICROSCOPY (SEM) EXAMINATION OF FRUIT STOMATA.

Objective: Developing strawberry fruit were examined for the presence of stomata.

Protocol. Portions of green, green-white, and red 'Brighton' fruit were fixed under a vacuum of 15 inches of Hg in FAA for 3 h, then moved through a 50%, 70%, and 100%/trichlorotifluroethane series at 30 min changes. The samples were put in a Balsers CPD 020 dryer and critical point dried, mounted on aluminum mounts with DUCO cement (Devcon Corp., Woodale, IL.) then coated with 20 nm of 60:40 wt% Au/Pd alloy in Varian VE vacuum evaporator operating at 1 x 10^{-5}Torr. Examination was made at 10 KV in an AmRay 1000A scanning electron microscope. Images were recorded on Polaroid Type 55 P/N film.

Conclusion: We failed to identify stomata on the achenes or fruit surface at green, green-white, or red stages of fruit development (see Figs. E1, E2, and E3).

Figure E1. Scanning electron microscopy (SEM) photograph (100X) of a 'Brighton' strawberry fruit and achene surface at the green stage of development.

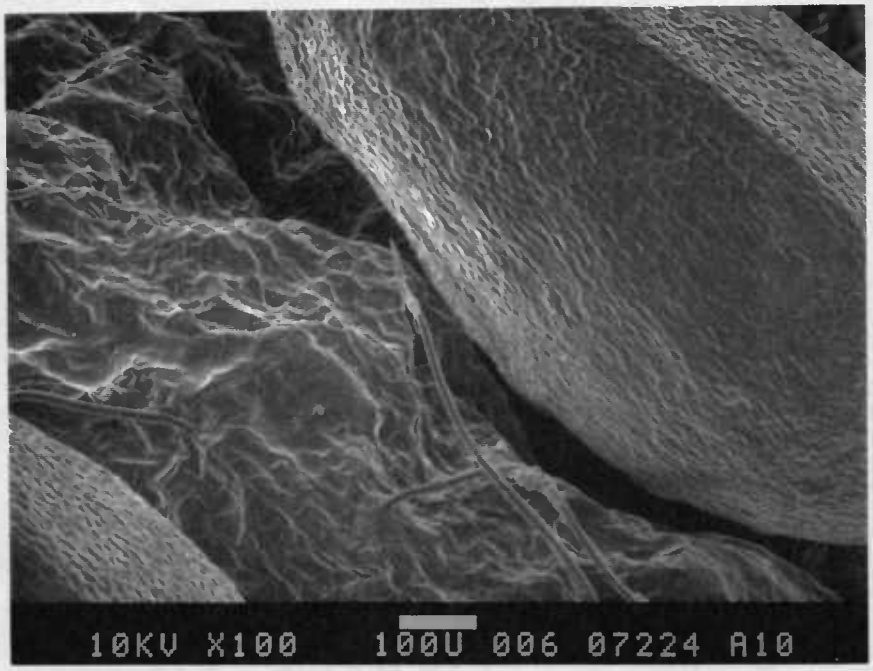

Figure E2. Scanning electron microscopy (SEM) photograph (100X) of a 'Brighton' fruit and achene surface at the green-white stage of development. Trichomes can also be seen on the fruit surface.

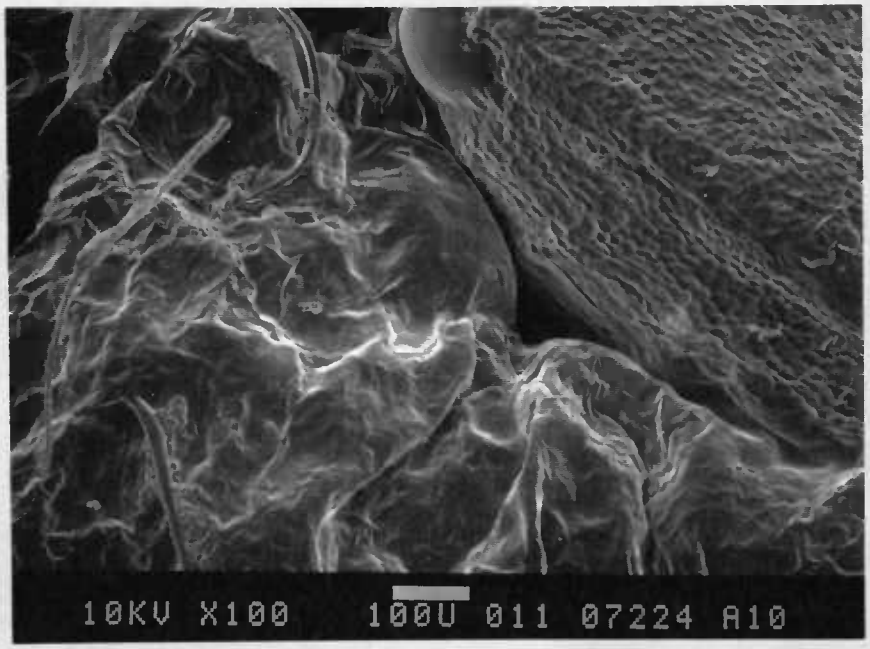

Figure E3. Scanning electron microscopy (SEM) photograph (100X) of the fruit and achene surface of a red `Brighton' strawberry fruit. Trichomes are evident on the fruit surface.

APPENDIX F

ANALYSIS OF VARIANCE FOR DATA PRESENTED IN CHAPTER 3

Table F1. One way ANOVA for Randomized Complete Blocks (days).
Variable: Solute potential

		leaf		red fruit
Source	df		Mean square	
Day	2	0.008^{NS}		0.017^{NS}
Main effect stress	1	0.112*		0.026^{NS}
Error	2	0.002		0.004
Total	5			

*significance at p = 0.05; NS = not significant

Table F2. Two Way Repeated Measures ANOVA on Two Factors.

Source	df	Leaf water potential	Fruit water potential	Difference between
		Mean square		
Day	3	0.521	0.410	0.058
Stress	1	6.848*	1.515^{NS}	2.375*
Stress x Day	3	0.054	0.371	0.177
Time	2	5.287*	1.450*	1.215
Time x day	6	0.211	0.135	0.103
Stress x time	2	0.177^{NS}	0.098^{NS}	0.425*
Residual	6	0.107	0.138	0.057
Total	23			

*significance at p = 0.05; NS = not significant

Table F3. Two Way ANOVA Split Plot

Source	df	Solute Potential	Volume change
		Mean Square	
Block	2	0.021	0.0006
Stress	1	0.232*	0.0300*
Main plot error	2	0.001	0.0010
Temperature	1	0.005^{NS}	0.014>*
Temp x stress	1	0.002	0.0001^{NS}
Error	4	0.010	0.0005
Total	11		

*significance at p = 0.05; NS = not significant