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dissertation entitled

Regulation of Carbohydrate Metabolism in Asparagus Spears (*Asparagus officinalis* L.)

presented by

Silvanda de Melo Silva

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Horticulture

Major professor

Date July 30, 1998

DATE DUE	DATE DUE	DATE DUE

REGULATION OF CARBOHYDRATE METABOLISM IN ASPARAGUS SPEARS (*Asparagus officinalis* L.)

By

Silvanda de Melo Silva

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1998

ABSTRACT

REGULATION OF CARBOHYDRATE METABOLISM IN ASPARAGUS SPEARS (*Asparagus officinalis* L.)

By

SILVANDA DE MELO SILVA

Sucrose depletion in harvested asparagus (*Asparagus officinalis* L.) spear tips is rapid and may trigger senescence. This research focuses on investigating how carbohydrate metabolism in asparagus spear tips, stored at 1 °C, is regulated. The research was divided in two parts. The first portion was an investigation into the effects of low O_2 and high CO_2 on respiratory and fermentative metabolism, sucrose-metabolizing and glycolytic enzymes, and phosphate and adenylate levels in spear tips. The second portion was an evaluation of the effects of exogenous cytokinin on sucrose metabolism in harvested asparagus spears.

For this portion of the research, spears were stored with a range of O_2 partial pressures combined with CO_2 treatments of 0, 5, 10, and 20 kPa using a system combining modified atmosphere packages in flow-through containers for a period of 6 days. The respiratory quotient increased and ethanol, acetaldehyde, and lactate accumulated below 2 kPa. During 6 days of storage, sucrose declined markedly, relative to harvest. Sucrose content was highest for spears exposed to 5 kPa CO_2. Sucrose synthase activity was higher at harvest; however, bound acid invertase activity was the highest after the storage period, indicating that sucrose may be degraded postharvest mostly in the apoplast. 5 kPa CO_2 reduced sucrose synthase, invertase and hexokinase activities, which may account for the reduction in sucrose utilization. Alcohol

dehydrogenase (ADH) and pyruvate decarboxylase activities increased for O_2 levels below 2 kPa. Lactate dehydrogenase activity was lower than ADH activity; however, it also increased below 2 kPa O_2. Visual quality (VQ) was reduced by CO_2, but increased as O_2 increased. VQ was best for spears stored in near-ambient O_2 and 5 kPa CO_2.

Low O_2 enhanced the interconversion of phosphoenolpyruvate (PEP) to pyruvate (PYR) and F6P to fructose-1,6-bisphosphate ($F1,6P_2$), indicating a promotion of glycolysis with a resulting loss of carbon in the sugar pool. Low O_2 caused an increase in pyruvate kinase (PK) activity. However, high CO_2 besides having an effect on PK, appeared to affect conversion of F6P to $F1,6P_2$ at O_2 levels below 2 kPa.

PPi and ATP decreased below 2 kPa O_2, with a concomitant increase in Pi, ADP, and AMP. Low O_2 also reduced adenylate energy charge (AEC) relative to high O_2. Higher CO_2 reduced AEC for O_2 levels greater than 2 kPa. Decreases in PPi, ATP, and AEC, and increases in Pi, ADP, and AMP in response to O_2 deficiency suggest impairment of oxidative phosphorylation and unbalanced cell metabolism, which may limit asparagus spear survival. Below 2 kPa O_2 the cells were under stress leading to fermentation. Below 1 kPa O_2 the spear tip tissues were experiencing severely limiting energy availability.

6-BAP reduced respiration rate of spears with intact tips, slowed changes in fluorescence, and slowed chlorophyll degradation during 31d of storage at 1°C. 6-BAP also slowed the decline in sucrose content in spears with tips. The visual quality was highest for spears with tips that were treated with 6-BAP.

To my parents José Jorge and Eliza

(*In memoriam*)

To my husband, Djail and my son, Lucas,

for their love and support

ACKNOWLEDGMENTS

I would like to express my gratitude to my major professors, Dr. Robert Herner and Dr. Randy Beaudry. Dr. Herner provided me with guidance, enthusiastic support, encouragement, and friendship throughout my graduate program. Dr. Beaudry supported me with friendship and enlightening guidance. His sharp vision in pursuing new research avenues provided a foundation for my scientific development and is genuinely appreciated.

I am grateful to the members of my guidance committee: Dr. Wayne Loescher, whose vision and advices greatly improved this work; Dr. Ken Poff and Dr. Mark Uebersax, for their constant encouragement and for bringing insightful suggestions into the proposed research. In addition, I would like to thank Dr. D.R. Dilley. His scientific rigor will remain as an example for my career.

A special thanks goes to Dr. John Everard for his valuable technical expertise and for his constructive comments and suggestions. This work benefited greatly from his knowledge and willingness to help. I also wish to thank the Postharvest group, especially Dr. Dina Kadyrzhanova and Dr. Nazir Mir, for for their constant help.

I also wish to thank Michigan State University for providing me the unique opportunity to participate in the doctoral program in Postharvest Physiology offered by the Department of Horticulture. The financial support of the Ministry of Education of Brazil, through the CAPES (Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) fellowship program and Universidade Federal da Paraíba (UFPB) made possible the completion of my degree.

I thank my graduate fellows in the Department of Horticulture, especially Stacie Wee, Jennifer Dwyer, and Rijida Leepipattanawit for their valuable friendship, constant support and encouragement.

I would like to thank to the Main Office of the Department of Horticulture, specially to Ms. Joyce Jackson, Sherry Mulvaney and Lorri Busick for their assistance.

I want to thank my friends and colleagues at UFPB, especially Rita de Cássia Queiroga, Roberto Germano Costa, Arinaldo Frazão, and Genival Azeredo for their encouragement and support. I wish to thank the many friends of the Brazilian community at Michigan State University. Special thanks goes to José Lima and family, for their friendship and precious help in getting started at MSU; Dorotéia Paglis and family, for their friendship and for being close by.

Finally, I wish to thank my sister (Cirlene) and brothers (Cícero, Cidene, Bartolomeu, Civaldo, e Civanildo) for always being there and believing in me.

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INTRODUCTION

Harvesting and handling of horticultural crops can impose a series of stresses, including wounding, dehydration and nutrient deprivation. Harvest stresses are particularly severe on organs that are actively growing at harvest. Harvested actively growing tissues tend to senesce rapidly due to an inability to maintain metabolic homeostasis (Davis and King, 1993).

Harvested asparagus spears deteriorate rapidly, having a shelf-life of only 3-5 days at room temperature (Lipton, 1990). The spear tip is composed of mostly meristematic tissue and is usually the first portion of the harvested spear to deteriorate (Davis and King, 1993). This postharvest deterioration is accompanied by numerous physiological and biochemical changes (Lipton, 1990). Following harvest, the level of soluble carbohydrates, especially sucrose, decline rapidly, the respiration rate declines markedly, protein is lost, free amino acids increase (specially asparagine) and ammonia can accumulate (Hurst et al., 1993; King et al., 1990). Indeed, sucrose content declines approximately 60% within 6 h after harvest at 20 °C (Irving and Hurst, 1993), and changes in gene expression also occur very early, including *de novo* induction of specific genes (King and Davis, 1992).

Postharvest research is largely directed at maintaining quality and prolonging the storage life of harvested whole and minimally processed crops. In many cases the fundamental physiological and biochemical processes that result in changes in quality are poorly understood. Many of these processes are inter-related or interdependent.

Improving our understanding of the fundamental processes or steps that exert control over factors that limit quality will likely provide opportunities for improving storability.

Glycolysis is an ubiquitous pathway that operates under both aerobic and anaerobic conditions (Kennedy et al., 1992) and its central importance in the generation of energy and the regulation of carbon flux has obvious implications on storability. Reduced O_2 levels and/or elevated CO_2 can alter glycolysis via effects on respiration rate (Kader, 1986). Lowering O_2 partial pressures too far, however, may induce the fermentative pathway. If O_2 levels are sufficiently low, fermentation can replace the Krebs cycle as the main source of metabolic energy. The relatively energy inefficient fermentative metabolism may alter cellular energetics. Other undesirable physiological changes, such as off-flavors may also occur (Ke et al., 1993). Understanding the physiology of sucrose metabolism, the role of glycolytic enzymes, and changes in energy charge should elucidate how harvested asparagus spears coordinate carbohydrate partitioning, metabolic capacity, and carbohydrate utilization processes.

The main focus of this research is to investigate how carbohydrate metabolism is regulated during storage in harvested asparagus spears. To accomplish this, the research project was divided into four main topics: 1) determining how utilization of sucrose and carbon flux govern fermentative metabolism; 2) evaluating the implications of reduced levels of O_2 and elevated CO_2 on carbon flow and the glycolytic pathway; 3) evaluating the impact of reduced O_2 and elevated CO_2 on phosphate and adenylate energy supply; and 4) evaluating the effects of exogenous cytokinin on sucrose metabolism in harvested asparagus spears.

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CHAPTER 1

LITERATURE REVIEW

Numerous physiological and biochemical processes are involved in metabolism of harvested asparagus spears. Many of those processes directly impact spear quality and storability. The acquisition and storage of energy and the utilization of this stored energy are central processes in the control of the plant metabolism. In intact plants, there is an interdependence between organs that supply and utilize energy. The harvest disrupts this interdependence and may, therefore, influence postharvest behavior. For example, acquisition of energy through photosynthesis and utilization via respiration are opposing forces in the intact plant, and photosynthesis does not occur in all products after harvest. To understand these distinct and interdependent processes and their roles in supporting life of harvested asparagus spear, carbon allocation, sucrose metabolism, and respiration need to be assessed simultaneously and described in detail.

Asparagus Botanical Background

Asparagus (*Asparagus officinalis* L.) is grown for its succulent fleshy shoots (spears), which appear after a winter resting period. Asparagus is an European-Siberian continental plant related to the East Mediterranean vegetation. It is a monocotyledonous (Robb, 1984), member of the Liliaceae (lily) family that has been cultivated in Europe and Asia for more than 2000 years (Zandstra et al., 1992). The common name and the Latin name are identical. 'Officinalis' means 'medicinal', a property for which asparagus was first noted. (Nonnecke, 1989). Cultivated asparagus is a diploid (n=10) species (Robb,

1984). Asparagus is dioecious (male and female flowers occur in separate plants) with a sex ratio of 1:1 (Reuther, 1985). However, male plants have flowers that vary from staminate (no functional carpels) to hermaphroditic (Robb, 1984). New all-male hybrids plants produce higher spear yield (Zandstra et al., 1992).

Biology of Growth and Carbon Allocation

Asparagus is an herbaceous perennial, and the aboveground foliage is known as the fern, the underground rhizomatous stem is termed the crown (Nonnecke, 1989). The spears develop into fern-like shoots, each of which developed from a separate bud on the underground rhizomatous stem. Each shoot consists of a central stem which subtends many fine, needle-like branches or cladophylls. The leaves in the cladophylls have been reduced to very small scales (Pressman et al., 1989).

As in other monocotylenoneus species, the crown of the asparagus plant is the critical growth center (Robb, 1984), in which fructo-oligosaccharides and fructo-polysaccharides (fructans) are the storage carbohydrates (Shiomi, 1992). The roots of the plant consist of underground stems (rhizomes), fleshy roots, and fibrous roots. The fleshy roots serve as storage organs and the fibrous roots as absorption organs. The fibrous roots die after each year's growth (Nonnecke, 1989). The fleshy roots remain to provide nourishment for the next generation of spears. During the fall and winter, low temperatures induce dormancy in the asparagus plant, accompanied by die-back of the mature fern. With the coming of the spring and the end of dormancy period, the fructan content in the crown decreases with the end of dormancy period concomitant with spear (young shoot) production and harvest. Growth of new spears is presumed to be at the

expense of previously stored carbohydrates, which are replenished later in the season following canopy development and maturation (Pressman et al., 1989). Although fructans are mostly fructose polymers, feeding $^{14}CO_2$ to tissue accumulating fructans leads to the initial appearance of radioactivity in sucrose, followed by the progressive appearance of label in fructans (Pollock et al., 1996). The fructans are synthesized from sucrose via a trisaccharide intermediate, by the combined action of two fructosyl tranferases: 1. sucrose:sucrose fructosyl transferase (SST, EC 2,4.1.99) and 2. fructan:fructan fructosyl tranferase (FFT, EC 2.4.1.100). In asparagus crowns, fructans account for 85% of the total carbohydrates, with sucrose and smaller oligofructans representing the remaining 15%. Of fructans, approximately 91% are polymers containing five or more fructose units (Cairns, 1992). Of the remaining 9%, the degree of polymerization generally ranges from 12 to 21 fructose units (Shiomi, 1993). These longer chain fructans consist mainly of iso-kestose and neo-kestose (Shiomi, 1992). The presence of sucrose and small oligofructans in the crown suggests that the crown, in addition to being a storage organ, may serve as a passage for the assimilate stream between the above and underground part of the plant (Martin, 1990). In spears, fructose, glucose, and sucrose are the predominant carbohydrates (Irving and Hurst, 1993). Fructans have been detected in the spear in some studies (Martin, 1990), but not in others (Pressman et al., 1989; Silva et al., 1998). Upon maturation of the shoot, fructan content in the roots increases again.

Early products of the photosynthetic carbon reduction (PCR) cycle in the chloroplasts are phosphoglycerate and dihydroxyacetone phosphate. These sugar phosphates are biochemically and spatially partitioned between two major biosynthetic

pathways, one leading to the synthesis and then retention of carbohydrate reserves, such as starch in plastids, and another to sucrose synthesis in the cytoplasm (Gifford et al., 1984). Asparagus has a C3 phothosynthetic pathway and the cladophylls are asparagus's main site of carbon assimilation. Carbon assimilation is very dependent on the amount of solar energy available, and the optimum temperature for photosynthesis is about 18 °C (Sawada et al., 1962). The yield of asparagus is not directly the result of current photosynthesis but is instead a function of carbohydrate reserves, produced by the previous year's ferns (Downton and Törökfalvy, 1975). Thus, yield is largely determined the year prior to harvest.

Carbon Metabolism in Harvested Asparagus

Sucrose is the primary transport form of reduced carbon in many higher plants (Huber and Akazawa, 1986). Thus, it is necessary to understand the pathway of sucrose metabolism as a means to understand processes regulating energy production and carbon flux at the biochemical level. Asparagus spears utilize imported sucrose to provide energy and carbon skeletons for growth. In the spear, the tip is the most metabolically active zone and is the strongest carbohydrate sink, which demand more translocated assimilates. The base of the spear, however accumulates carbohydrates and is a storage sink (Hurst et al., 1993). In asparagus spears, high levels of sucrose at harvest (Irving and Hurst, 1993; Silva et al., 1998) together with the absence of fructans in the spears (Pressman et al. 1989, Silva et al., 1998) suggest that sucrose is the major carbohydrate being translocated from roots to spears and, during carbon assimilation, from the spears to the roots. In harvested asparagus, sucrose movement has been suggested to be from the base to the

upper parts of spears (Saltveit and Kasmire, 1985). Harvesting asparagus immediately removes the source of respiratory substrates and, due to its very high metabolic rate (Irving and Hurst, 1993), leads to rapid loss in carbohydrate. After 7 d storage at 1 °C, the respiratory demands of spear tips had outstripped the rate of use of soluble carbohydrate available in that tissue, suggesting that substrates are transported from the base to the tip during postharvest storage or that material other than sugars may be used to sustain metabolism (Silva et al., 1998). Such rapid depletion in sugars apparently leads to carbohydrate starvation typically seen in senescing or sugar-starved plant tissues (Brouquisse et al., 1991). Other studies on sycamore cells and excised maize root tips, have provided evidence that cell contents are degraded to sustain metabolism during carbohydrate starvation. In those tissues, loss of carbohydrates, proteins and lipids was accompanied by an increase in free amino acids, inorganic phosphate, and phosphorylcholine, and reduction in activity of glycolytic enzymes (Dorne, 1987; Genix et al., 1991). The decline in sucrose content in asparagus is accompanied by a drop in respiration rate; these are two of the earliest physiological events detected after harvest (Irving and Hurst, 1993; Silva et al., 1998). Sucrose depletion is suggested to trigger senescence (King et al., 1993). Thus, the sucrose-metabolizing enzymes may control sucrose decline in harvested spears. Understanding the role and regulation of enzymes in sucrose catabolism could provide a means to control loss of sucrose and, therefore, extend the shelflife of asparagus.

Sugar-regulated genes provide a means for the plant to integrate the cellular response to transport sugars and to coordinate changes in resource utilization and

allocation among parts. To this end, genes upregulated by increased carbohydrate availability in plants include many that would enhance sucrose utilization through storage, respiration, and biosynthetic processes (Koch, 1996). Also, a major contribution to regulation of carbon partitioning is the expression of genes of the sucrose-metabolizing enzymes (Geiger et al., 1996). In addition, glucose and fructose from sucrose breakdown are thought to repress the transcription of photosynthetic genes in plants (Sheen, 1990), probably acting via a hexose sensor (Jang and Sheen, 1997; Jang et al., 1997).

In asparagus, sugar depletion may also regulate the expression of genes involved in specific carbon and nitrogen remobilization processes (King et al., 1995; King and Davis, 1995). In response to carbohydrate stress, asparagine synthetase (EC 6.3.5.4) mRNA levels begin to increase in asparagus tip tissues within 2 h following harvest (King and Davis, 1995). The increase in asparagine synthetase message precedes the accumulation of asparagine, which occurs approximately 6 h after harvest King and Davis, 1995). Asparagine accumulation is considered to be a postharvest senescence symptom (Eason et al., 1996). During senescence, visual appearance of the spear changes; symptoms include feathering and browning of bracts, tissue flaccidity and cellular breakdown (King et al., 1990).

Sucrose Metabolism

Photoassimilate partitioning to sinks is a highly regulated process (Stitt and Sonnewald, 1995) that includes sugar transporters (Kruger, 1990) and sucrose-metabolizing enzymes (Sturm, 1996) (Fig. 1). Sucrose and starch are two of the most important end-products of photosynthesis (Quick and Schaffer, 1996). Sucrose, however,

is the major form in which carbon is translocated (Stitt and Sonnewald, 1995) in addition to being an important storage carbohydrate in plants (Ehness and Roitsch, 1997). Sucrose transport in the phloem is suggested to be driven by a turgor pressure gradient generated by a sucrose concentration gradient, with high sucrose concentrations at the site of phloem loading and lower concentrations at the site of phloem unloading (Ho and Baker, 1982). Transport of sucrose from the apoplast into the phloem is catalyzed by a H^+/sucrose co-transporter of the companion cell plasma membrane and driven by ATP hydrolysis at the cytoplasmic side of the transport protein (Daie, 1989).

Once translocated, sucrose enters in the sink cells by either crossing the plasma membrane apoplastically via sucrose carrier or by diffusion, and/or sucrose enters symplastically through plasmadesmata (Krausgrill et al., 1996; Sung et al., 1989). If sucrose is the primary form in which carbohydrates are stored, high sugar levels may be favored in the cell because sucrose contributes half of the osmolarity of the equivalent of glucose and fructose (Steingröver, 1983) and may be less metabolically accessible to respiratory loss than hexose sugars (Sung et al., 1989). As proposed by Ho (1988), the flow of sucrose into a tissue (sink strength) is regulated by the rate of sucrose degradation. In carrots, sink strength is generated by a common action of sucrose synthase and vacuolar invertase and, especially in the case of apoplastic unloading, by the active transport of sucrose across the membrane (Sturm, 1996). It is through sucrose degradation that glycolysis is initiated in most plants (Kruger, 1990).

Two pathways for the degradation of sucrose exist and provide the basis for a flexible system that can markedly affect the partitioning and metabolism of assimilated

carbon in sink tissues (Huber and Akazawa, 1986) (Fig. 1.1). Sucrose can be degraded and metabolized in higher plants by sucrose synthase (SUSY, EC 2.4.1.13) or invertases (ß-fructosidase, EC 3.2.1.26). Specific sucrose-metabolizing enzymes are associated with specific sink functions. For example, invertases predominate in storage sinks while SUSY predominates where cell wall expansion is active (Sung et al.,1989).

Sucrose metabolism is responsive to developmental patterns in cell division and differentiation. During rapid cell wall biosynthesis, the incoming sucrose is preferentially channeled to cellulose (Delmer and Amor, 1995). In storage tissues, sucrose is largely diverted to carbohydrate reserve and/or soluble sugars (Krausgrill et al., 1996). In these tissues sucrose is transported into the vacuole (Kruger, 1990). In storage tissue sucrose hydrolysis to fructose and glucose is by vacuolar invertase (Krausgrill et al., 1996); the hexoses produced are part released in the cytoplasm and part retained in the vacuole.

Invertase hydrolysis of sucrose to hexose sugars plays a fundamental role in the energy requirements for plant growth and maintenance (Klann et al., 1996). The reaction is highly exothermic and irreversible (Avigard, 1982; Kruger, 1990). The functions of invertases in sink tissues are likely complex as reflected by the existence of differentially expressed isoforms (Sturm et al., 1995), some of which are inactivated by inhibitory proteins at certain stages of plant development (Greiner et al., 1998; Krausgrill et al., 1996). Isoforms may differ in their cellular location (vacuole, cytoplasm, apoplast), their spacial expression during development (Sturm et al., 1995), and their expression in the presence of different glucose concentrations as in 'feast' and 'famine' isoforms of vacuolar invertase in maize (Koch et al., 1996). In addition, specific isozyme genes are also

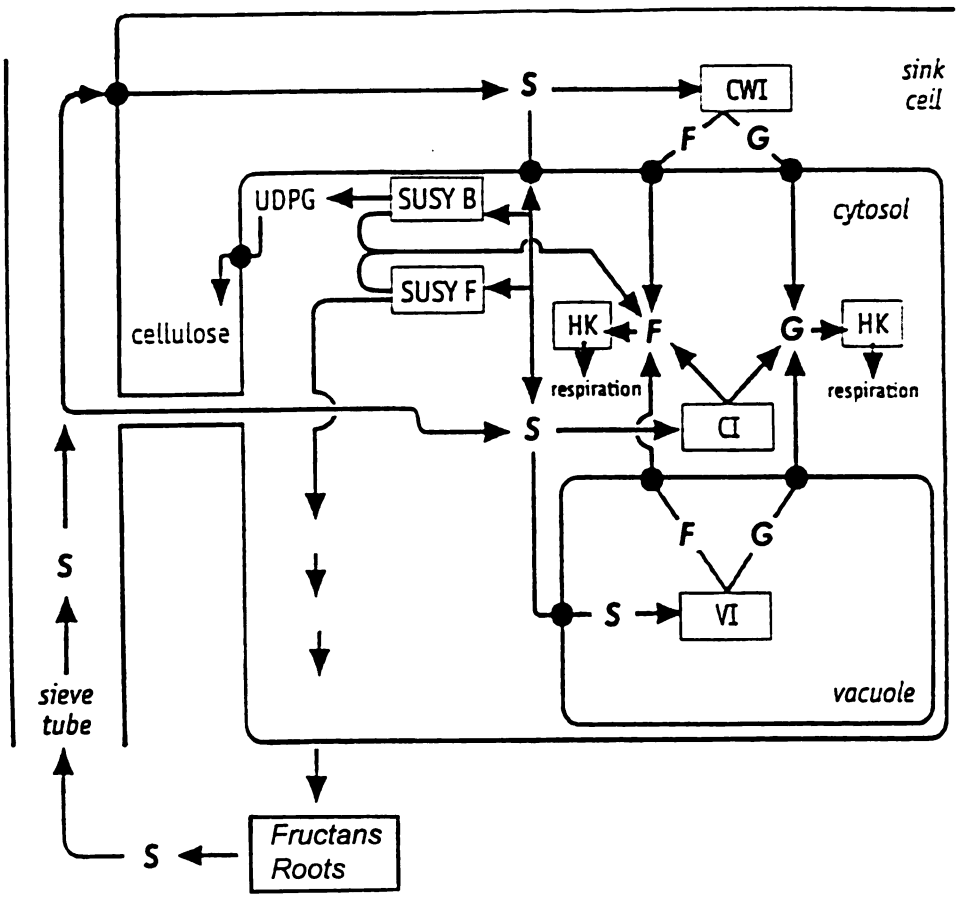

Figure 1.1. Proposed cellular pathways of sugar transport and metabolism in a sink cell of asparagus supplied with sucrose via symplastic and apoplastic routes. Enzymes are: bound sucrose synthase (SUSY B) and soluble sucrose synthase (SUSY F), invertases (VI, cytosolic neutral; VI, vacuolar acid; CWI, cell wall bound acid), and hexokinase (HK). Substrates are sucrose (S), glucose (G), and fructose (F). Sugar transporters and cellulose synthase are represented by black dots. Adapted from Krausgrill et al. (1996).

expressed when carbohydrate supplies are limited. For example, *Chenopodium rubrum* invertase isozyme genes have at least three members (Ehness and Roitsch, 1997).

There are two types of acid invertases in plants: vacuolar soluble (VI) and cell wall-bound (CWI) invertases, which have optimum activity about pH 5.0, and are present in the vacuole and in cell wall, respectively. Cytosolic (neutral) invertase (CI), also termed alkaline invertase, has optimum activity at pH 7.0-7.5 and is localized in the cytoplasm (Klann et al., 1996). High activity of CWI, which is ionically bound to the cell wall, is usually associated with rapidly growing tissues, where these enzymes are said to play a major role in maintaining the source-to-sink gradient in sucrose concentration by rapidly hydrolyzing incoming sucrose (Krausgrill et al., 1996). CWI has been proposed also to be important for phloem unloading and sink strength (Ho, 1988). CWI has been cloned and it has been shown that the mRNA levels increases in response to sugars (Karrer and Rodriguez, 1992), giving further evidence that invertases also belong to the group of enzymes that are under metabolic control by carbohydrates. CWI expression is often high in young growing tissues (Sturm et al., 1995). However, it seems that high CWI activity is not necessarily linked to apoplastic sucrose unloading from the phloem. Rather it seems that even in symplastically supplied sinks, a considerable amount of sucrose may diffuse from the sink cells and be hydrolyzed in the apoplast (Krausgrill et al., 1996). VI has a higher activity in the rapidly elongating region of the asparagus spear than in the more apical or basal tissues, which is 6 cm below the spear tip (Hurst et al., 1993).

SUSY catalyzes an equilibrium reaction that degrades sucrose to UDP-glucose and fructose and so conserves energy equivalent to one ATP in the glycolytic bond

relative to invertase (Geiger et al., 1996). UDP-glucose can be converted to respiratory intermediates by being converted to glucose-1-P or glucose and used throughout respiration (Kruger, 1990), the UDP-glucose generated from sucrose by SUSY is a intermediate in the biosynthesis of cell wall polysacharides, amino acids, lipids, etc (Sturm, 1996). SUSY activity is three times higher than neutral invertase in asparagus spear tips at harvest (Irving and Hurst, 1993). High SUSY activity has also been reported to be present in other rapidly growing tissues (Ap Rees, 1984; Avigard, 1982; Geigenberg and Stitt, 1993), and it has been suggested that this enzyme activity can be used as a measure for sink strength (Sung et al., 1989). SUSY is localized in the cytosol, or attached to the inner side of plasma membrane (Delmer and Amor, 1995).

Sucrose synthase, in contrast to invertase, catalyzes a freely reversible reaction, with a theoretical equilibrium constant (Keq) in the direction of sucrose degradation ([UDP-glucose] [fructose])/([sucrose]) of 0.15-0.56 (Morell and Copeland, 1985). Enzymes that catalyze irreversible reactions are generally considered good candidates for regulation. Conversely, enzymes that catalyze near-equilibrium reaction are considered to be in excess and, hence, poor candidates for regulation (Stitt, 1990). However, a readily reversible reaction can be rendered effectively irreversible in vivo by removing one of the products. The reaction catalyzed by SUSY may be displaced from equilibrium *in vivo*, due to the rapid removal of fructose or UDP-glucose. For instance, in intact growing potato tubers, the SUSY reaction may be displaced from equilibrium in favor of sucrose degradation (Geigenberger and Stitt, 1993). The flux through SUSY responds promptly to the supply of sucrose and the demand of sucrose in the cell.

Tissues that have high metabolic activity such as asparagus spear tips have a high demand for sucrose in order to maintain the carbon supply for respiratory and biosynthetic pathways. In sycamore cells, SUSY causes the rate of sucrose degradation to respond sensitively and automatically to those demands (Huber and Akazawa, 1986), but, at least in potato tubers, SUSY does not regulate the rate of sucrose degradation (Geigenberger and Stitt, 1993). In some cases, SUSY may operate in parallel with irreversible reactions catalyzed by sucrose phosphate synthase or invertase (Xu et al., 1989).

In the case of sucrose being metabolized by invertase, it is essential that this reaction is regulated. High invertase activity could inhibit sucrose movement into a sink, because one molecule of sucrose is replaced by glucose and fructose, two osmotically active products (Geigenberger and Stitt, 1993). If these hexoses accumulate to significant levels, they will alter the regulation of turgor and water movement within the symplast and between symplast and apoplast (Heineke et al., 1992). Hence, sucrose metabolization via invertase will presumably require sophisticated regulatory mechanisms to coordinate invertase and hexokinase (EC 2.7.1.1)/fructokinase (EC 2.7.1.4) activity with cell growth and turgor (Geigenberger and Stitt, 1993; Kruger, 1990). The two latter enzymes are also suggested as having a glycolytic regulatory role *in vivo* (Jang et al., 1997). SUSY plays a critical role in anoxic tolerance in maize seedling roots, since it is the main enzyme for metabolizing sucrose under O_2 deprivation (Ricard et al., 1998). However, it seems that different genes and signal mechanisms for SUSY are expressed under hypoxia and anoxia, in a manner that parallels the induction of "feast and famine" SUSY genes by hypoxia and anoxia, respectively (Zeng et al., 1998).

There are analogies between SUSY and another unusual enzyme in plant metabolism, pyrophosphate:fructose-6-phosphate phosphotransferase (PPi:PFK, EC 2.7.1.90). Both of these enzymes are PPi-dependent, and catalyze a readily reversible reaction which, in effect, short-circuits an irreversible reaction. Furthermore, both enzymes are highly regulated. SUSY is inhibited by free glucose and fructose, and also shows large changes in activity during development and in response to sugars (Farrar, 1991), presumably due to regulation at the level of gene expression (Maas et al., 1990) and by post-transcriptional mechanisms (Doehlert and Chourey, 1991). PPi:PFK is regulated by substrates and products, and fructose-2,6-bisphosphate, and also undergoes developmentally regulated changes, being higher in metabolically active tissues (Stitt, 1990).

Reduced levels of O_2 and/or elevated levels of CO_2 are known to affect respiratory and enzyme activities in plant tissues (Kato-Noguchi and Watada, 1996a, b; Kerbel et al., 1988). Under anoxia, sucrose metabolism seems to take place mainly through SUSY in rice seedlings (Guglielminetti et al., 1995; Ricard et al., 1994), and maize, wheat and barley seeds (Guglielminetti et al., 1997). The degradation of sucrose may be reduced if asparagus spears are stored under limiting O_2 and CO_2 levels as a result of decreasing the activity of the enzymes involved in sucrose catabolism. Investigating the effect of O_2 and CO_2 on the metabolism of sugars in asparagus spear tips may be a way to identify by which means the carbon flux is regulated.

Respiratory Metabolism

Respiration is the anabolic process that involves the oxidative breakdown of carbohydrates, organic acids, lipids, and, on occasion, proteins to CO_2 and H_2O. This process results in concomitant generation of energy in the form of reducing power and ATP, and the formation of carbon skeletons to be used by the cell for biosynthetic reactions, maintenance of cellular organization and membrane integrity, preservation of ion gradients and protein turnover. This oxidative process can be subdivided in three stages: 1) glycolysis, 2) the tricarboxylic acid (TCA) cycle, and (3) the electron transport chain (Taiz and Zeiger, 1991). From a postharvest point of view, rate of respiration is important not only because of those processes, but also because it also gives an indication of the overall rate of metabolism of the plant or plant part (Kays, 1991).

Controlled atmosphere (CA), modified-atmosphere (MA), and modified atmosphere packaging (MAP) systems refer to techniques for creating gas mixtures differing from that of air and have been used as tools to minimize deterioration, improving quality in fruits and vegetables (Kader, 1986; Lakakul, 1994). Depending on the degree of atmosphere modification, there can be positive or detrimental effects to the commodities (Cameron et al., 1994). High levels of CO_2 can either slow or elevate respiratory rates depending on the commodity and concentration of CO_2 employed (Beaudry, 1993; Kerbel et al., 1988). An elevation of the respiratory quotient (RQ) can also take place due to CO_2-enhanced synthesis of ethanol and acetaldehyde (Thomas, 1925). The effect of CO_2 on the synthesis of fermentation products is further modulated by the presence of O_2 (Shirazi et al., 1991; Thomas, 1925). A variety of techniques have

been used to investigate the effects of reduced O_2 and elevated CO_2 levels on respiration (Baxter and Waters, 1991; Beaudry, 1993; Corrigan and Carpenter, 1993; King et al., 1986; Lakakul; 1994). The use of O_2/CO_2 barrier films may have potential for MAP of a perishable horticultural crop like asparagus. However, a film that matches the respiration rate of asparagus should be carefully tested to control possible anaerobic respiration during storage. Systems using a flow through design in which small differences between inlet and outlet gas concentrations are determined, permit analysis of O_2 and CO_2 on respiration, however the accuracy can be poor. On the other hand, modified-atmosphere packaging (MAP) systems as respirometers, like flow-through systems, achieve steady state conditions and allow the evaluation of the effects of a broad range of O_2 and CO_2. The fluxes of O_2 and CO_2 in a package system are calculated from the respiration-driven gradients of O_2 and CO_2 across the film (Beaudry et al., 1993). This technique allows establishing accurate ranges of O_2 and CO_2 in a packaging system, and also allows accurate respiratory measurements despite high backgrounds levels of O_2 and CO_2. In asparagus, however, much of CA and MAP research has been directed toward the determination of the effects and optimum conditions of lower O_2/ higher CO_2 for postharvest storage (Baxter and Waters, 1991; Corrigan and Carpenter, 1993; King et al., 1986; King et al., 1993; Lipton, 1965). Importantly, gas concentrations achieved in MAP under commercial conditions may differ significantly from optimal conditions (Cameron et al., 1994). The postharvest basis of using low O_2 and/or high CO_2 for storage of commodities has been assessed at biochemical (Kato-Nogushi and Watada, 1996a, b; Ke et al., 1995; Kerbel et al., 1988; Lange and Kader, 1997a, b, c) and physiological levels

(Beaudry, 1993; Cameron et al., 1994; Gran and Beaudry, 1993). However, biochemical studies have been restricted to 2 or 3 levels of O_2 and/or CO_2, which limits a broader evaluation of the processes involved. Furthermore, little is known regarding atmosphere effects on asparagus carbohydrate metabolism (Hurst et al., 1997).

Glycolysis. Glycolysis is carried out by a group of soluble enzymes located in the cytosol (Fig. 1.2). Glycolysis functions to fulfill two fundamental roles. It oxidizes hexoses to generate ATP, reductant, and pyruvate, and it produces carbon skeletons for synthetic reactions. Chemically, hexoses (derived primarily from starch, sucrose, fructans, and other sugars) undergo a limited amount of oxidation to produce two molecules of pyruvate, two molecules of ATP, and two molecules of the reduced pyridine nucleotide, NADH (Taiz annd Zieger, 1991). Thus, classical glycolysis is the cytosolic linear sequence of 10 enzymatic reactions that catalyze the net reaction:

Glucose + 2ADP + 2 Pi + 2 NAD^+ ------> 2 pyruvate + 2 ATP + 2 NADH [1].

Glycolysis is recognized as having a central role in carbon metabolism and is present, at least in part, in all organisms. Glycolysis is directly involved in many biochemical adaptations of plant and nonplant species to environmental stresses such as osmotic stress, drought, cold/freezing, hypoxia and anoxia (Plaxton, 1996). Glycolysis also can function in reverse to generate hexoses from various low-molecular-weight compounds in energy-dependent gluconeogenesis. Both processes are tightly regulated *in vivo* (Plaxton et al., 1996).

Glycolysis and gluconeogenesis, in conjunction with the pentose phosphate pathway, are at the central point of the allocation of carbon to the various biosynthetic

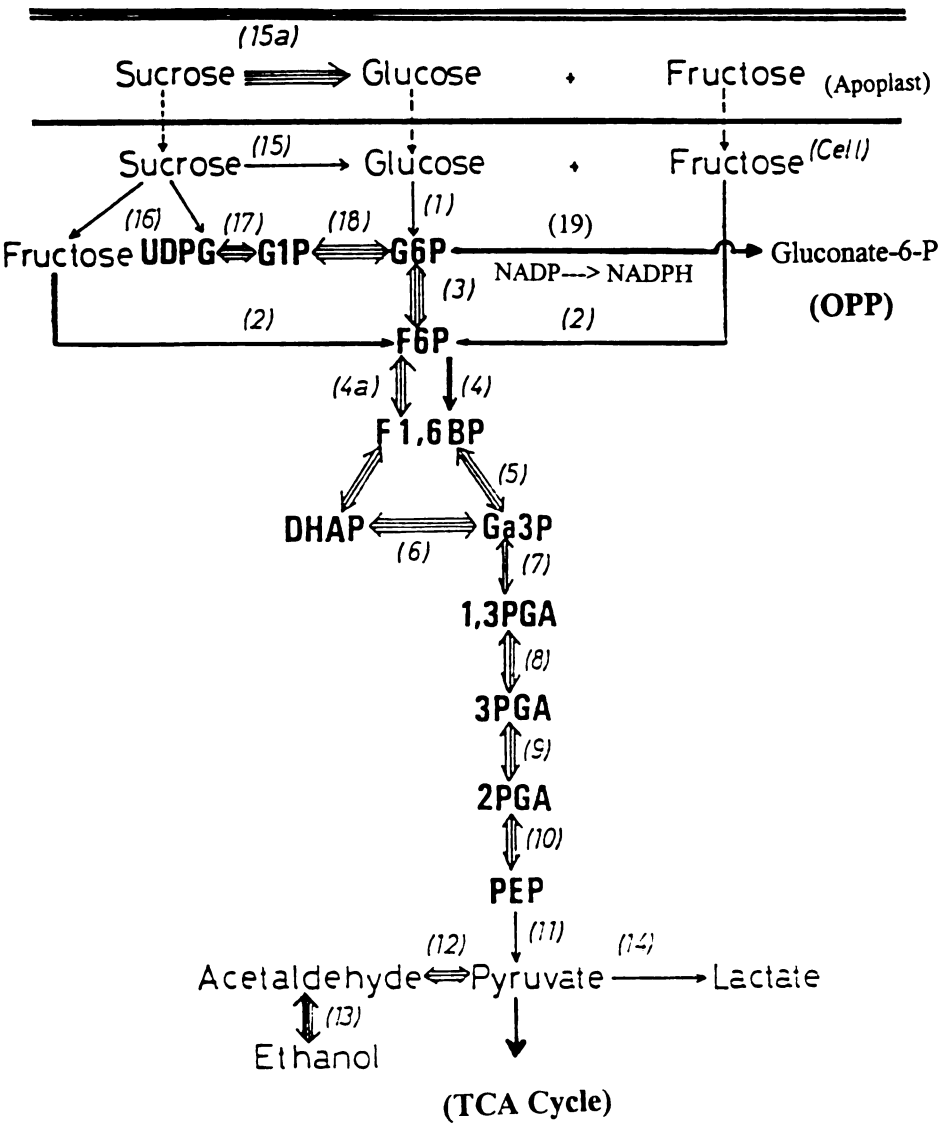

Figure 1.2. Glycolysis in plant tissues showing the relationship with oxidative pentose phosphate (OPP) pathway and TCA cycle. The numbers represent enzymes as follows: 1, hexokinase; 2 fructokinase; 3, phosphoglucoisomerase; 4, ATP:PFK; 4a, PPi:PFK; 5, aldolose; 6, triosephosphate isomerase; 7, glyceraldehyde-3-phosphate dehydrogenase; 8, phosphoglycerate kinase; 9, phosphoglycerate mutase; 10, enolase; 11, pyruvate kinase; 12, pyruvate decarboxylase; 13, alcohol dehydrogenase; 14, lactate dehydrogenase; 15, intracellular invertase; 15a, extracellular invertase; 16, sucrose synthase; 17, UDP-glucose pyrophosphorylase; 18, phosphoglucomutase; 19, glucose-6-phosphate dehydrogenase (Adapted from Ashihara et al., 1988).

pathways in plants. The metabolites comprising these pathways can be divided into a number of pools of phosphorylated intermediates and hexoses (Taiz and Zieger, 1991). The entry and exit of metabolites from pools are highly regulated, and this regulation is complicated by the existence of separate pools within the cytosol and plastids (Blakeley and Dennis, 1993). However, a fundamental characteristic of cytosolic glycolysis is the existence of two previously mentioned reactions that can utilize PPi rather than nucleoside triphosphates (NTPs) as a phosphoryl donor (Plaxton, 1996).

The ability to regulate the rates of metabolic processes in response to changes in the internal and or external environment is a fundamental feature that is inherent to plant material and allows the maintenance of an efficient functional state in all organisms (Plaxton, 1996). The flux of metabolites through any given pathway must be closely coordinated with the needs of the cell, tissue, or organism for the products of the pathway. A common way to accomplish such regulation is through altering the activity of at least one rate-limiting enzyme of the pathway (Blakeley and Dennis, 1993). The identification and characterization of key enzyme that function as rate limiting enzymes or pacemakers has been a major focus of research in glycolysis. The rate-limiting steps of a pathway are essentially irreversible (i.e., have high negative free energy) *in vivo*, have a low activity overall, and frequently occur at the first committed step of a pathway, directly before major branch points (Plaxton, 1990). The control analysis theory developed by Kacser and Burns (1973) attempts to provide a quantitative mechanism for probing intact biological systems and interpreting results without taking into account which enzymes were preconceived as 'rate-limiting steps' or 'pacemakers' in the pathway. These authors

have established the concept of the *flux of control coefficient* (C^J_E) whose value specifies the change in metabolic flux (ΔJ) to small changes in the activity of any enzyme (ΔE) in the metabolic system as follows:

$$C^J_E = (\Delta J) \cdot (\Delta E)^{-1} \quad [2].$$

Those enzymes with a control coefficient approaching 1 have a major control function in the pathway, while those with a coefficient approaching zero have little control. In this way, this theory assigns a flux control coefficient for each enzyme. In fact, according to Blakeley and Dennis (1993), it has become apparent that all enzymes of a pathway may exert some control on the flux. However, if one of the components of a pathway has a large C^J_E, flux through the pathway is particularly sensitive to small pertubations in the activity of that particular enzyme (Anderson, 1974).

'Coarse' and 'fine' metabolic controls are the two basic mechanisms potentially used by the cell to vary the reaction velocity of a particular enzyme. Coarse metabolic control is a long term (hours or days), energetically expensive response that is achieved through changes in the total cellular population of enzyme molecules. Coarse control may be important in long term environmental (adaptative) changes, such as imposition of lower O_2/higher CO_2 atmosphere. The reaction rate of a given enzyme is dependent on the rates of its biosynthesis versus degradation. Thus, any changes in the rate of transcription, translation, mRNA processing or degradation, or proteolysis contribute to coarse metabolic control (Plaxton, 1990).

Fine metabolic control often involves fast (seconds to minutes), energetically inexpensive regulatory devices which modulate the activity of the pre-existing enzyme

molecules (Cohen, 1983). Fine control are seen as "metabolic transducers" that sense the momentary metabolic requirements of the cell, and modulate the rate of metabolic flux through the pathways (Plaxton, 1990). There are several ways by which fine metabolic control can be exerted in plants: i) alteration in substrate or cosubstrate concentration, which modifies the of pathway flux, most significantly for enzymes that show sigmoidal substrate kinetics; ii) variation of pH, above or below the enzyme's maximal activity; iii) allosteric regulation, by which specific inhibitor or activator metabolites can reversibly bind in allosteric sites often present in multisubunit regulatory enzymes (adenine nucleotides are examples of allosteric regulators for ATP-PFK in plants); iv) covalent modification, which functions cooperatively with allosteric regulation and results in formation of stable covalent bonds, often by the action of two enzymes as in the case of dithiol-disulfide interconversion and phosphorylation-dephosphorylation (Loewe et al., 1996), which are reversible covalent modifications; v) subunit association-dissociation; vi) reversible associations of metabolically sequential enzymes. Essentially all rate-limiting enzymes are at least partially controlled by allosteric effectors (Plaxton, 1996).

Enzymatic analysis is a valuable tool for determining kinetics of the reactions and the influence of regulators. Often such work is necessary to demonstrate the existence of a particular enzyme or a pathway (Carnal and Black, 1979) and indicate the involvement in glycolysis. However, in many studies, the activity of glycolytic enzymes extracted directly from a tissue is used as a means to detect changes in enzyme activity during development or following a particular treatment. Such studies are subject to reasonable criticism when the attempt is made to extrapolate *in vitro* activity measurements to *in vivo*

situations (Kruger, 1995).

Quite often enzymatic analysis is supported by analysis of intermediate, substrate, and end-product contents or their production rates. Modification in the pattern of intermediate levels can be readily interpreted by comparing changes in the relative concentrations of successive intermediates along a biochemical pathway between two treatments. A convenient means to do this is via the crossover plot (Adams and Rowan, 1970; Chance et al., 1958; Ghosh and Chance, 1964), applying the following equation:

$$\text{Crossover value} = ([C_2] - [C_1]) \cdot (([C_2] + [C_1])/2)^{-1} \quad [3],$$

where C_1 and C_2 are initial and final concentrations of each intermediate at the start and end of a treatment. Changes in intermediate concentrations that cause the line connecting crossover values from two successive intermediate pools in a biochemical pathway to 'cross over' the zero line is interpreted to indicate a change in regulation and thereby indicating a regulatory enzymatic step. An increase in values from negative to positive means that the step was stimulated. Conversely, a decrease in values from positive to negative means that the step was inhibited. Use of the crossover theorem has proven to yield fairly consistent results in a variety of plant materials in that two glycolytic interconversions from fructose-6-phosphate to fructose-1,6-bisphosphate and from phosphoenolpyruvate to pyruvate are regularly recognized as major control points in the glycolytic sequence (Adams and Rowan, 1970; Kato-Nogushi and Watada, 1996; Kerbel et al., 1988). There are several regulatory interconversions in the glycolytic and pentose phosphate pathways:

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) is the key enzyme

controlling carbon flux into the oxidative pentose phosphate (OPP) pathway. Glucose-6-phosphate can flow either into glycolysis or through OPP (Taiz and Zieger, 1991). G6PDH catalyzes the oxidation of glucose-6-P to δ-glucono-1,5-lactone-6-phosphate with the concomitant reduction of $NADP^+$ (Stitt, 1990). In higher plants, elevated activity of G6PDH is associated with the production of NADPH for reductive biosyntheses, which may be regulated independently of glycolysis (Ap Rees, 1984).

Hexose kinase (HK) catalyzes the production of hexose monophosphates from glucose and fructose utilizing a nucleoside tri-phosphate (NTP) as a phosphoryl donor (Gardner et al., 1992). HK is thought to be the sensor in the signaling pathway in which glucose and other hexoses act to repress photosynthetic genes (Jang et al., 1997; Sheen, 1990). The phosporylation of hexoses by HK appears to be critical for signaling because only the hexose and glucose analogs that can be phosphorylated by HK are effective in signal transduction (Jang et al., 1997). There are a variety of hexose kinases in plant tissues (Turner and Turner, 1980). These enzymes may be subclassified on the basis of their substrate specificity. An enzyme that can effectively utilize several hexoses is termed hexokinase (HK, EC 2.7.1.1), while that which preferentially metabolizes glucose is termed glucokinase (GK, EC 2.7.1.2), and that which phosphorylates fructose is designated fructokinase (FK, EC 2,7.1.4) (Kruger, 1990). For example, in pea seeds, fructokinase fraction IV, was specific for fructose as a substrate, having little activity with glucose (Turner et al., 1977). In this case, this enzyme is probably responsible for the metabolism of fructose derived from the cleavage of sucrose by sucrose synthase (Kruger, 1990). In tomato fruits undergoing starch synthesis, FK is inhibited by fructose (Schaffer

and Petreikov, 1997). GK and FK phosphorylating activities is inhibited by anoxia in maize roots (Bouny and Saglio, 1996).

Three enzymes operate at the reversible interconversion of F-6-P to F-1,6-P_2. This step in carbon metabolism exerts control over glycolytic flux and is considered to be rating-limiting for glycolysis for some tissues/conditions. Pyrophosphate-dependent 6-phosphofructose 1-phosphotransferase (PPi-PFK, EC 2.7.1.90) catalyzes the interconversion of PPi and F-6-P to Pi and F1,6P_2 in plants (Smith et al., 1984). PPi:PFK was discovered in 1974 in amoeba (Reeves et al., 1974) and in 1975 in bacteria (O'Brian et al., 1975). PPi:PFK was later found to be present in plant tissue and exhibited similar regulatory properties (Carnal and Black, 1979). This reaction is fully reversible (Nielsen, 1995), however, PPi:PFK participates in glycolysis through the use of PPi both as a phosphate donor and as an energy source (Smyth et al., 1984). PPi:PFK can be interconverted between an active 260 kDa tetramer, and a less active 130 kDa dimer (Black et al., 1987). The state of aggregation of this enzyme is dependent on the concentrations of its allosteric activator, fructose 2,6-bisphosphate (F2,6P_2), and the product of the forward reaction, Pi (Plaxton, 1996). The activity of tetrameric form of the enzyme is enhanced by the presence of F2,6P_2 (Xu et al., 1984). Alternatively, PPi:PFK was demonstrated to be activated in barley leaves by F1,6P_2 in presence of aldolase (Nielsen, 1995). The dimeric form of PPi:PFK, however, as well as its activity in the reverse, or gluconeogenic direction, is promoted by Pi (Dennis and Greyson, 1987). PPi:PFK activity is inhibited by increased levels of Pi in the cytosol (Plaxton, 1996).

ATP:PFK (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11)

catalyzes the interconvertion of ATP and F-6-P to ADP and $F1,6P_2$ in higher plants and is essentially irreversible under physiological conditions (Plaxton, 1996). ATP:PFK operates in the glycolytic direction, in a non-equilibrium reaction. Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) functions gluconeogenically to catalyze the interconversion of $F1,6P_2$ to F-6-P. In contrast to PPi:PFK, FBPase is inhibited by $F2,6P_2$ (Plaxton, 1996). ATP:PFK and FBPase were called "maintenance enzymes" for the regulation of sugar metabolism (Black et al., 1987) due to their roles of remaining fairly stable in a given tissue throughout development for a variety of species.

ATP:PFK is under complex allosteric regulation. ATP is not only the substrate for ATP:PFK, but also inhibits its activity when present in high levels by binding to an allosteric site and lowering the affinity of the enzyme for its substrate F-6-P (Plaxton, 1996). High citrate levels increase the inhibitory effect of ATP, further reducing the carbon flux through glycolysis. In contrast, ADP and AMP, which rise in concentration when the consumption of ATP outspaces its production, act allosterically to relieve this inhibition by ATP (Dennis and Greyson, 1987). Phosphoenolpyruvate (PEP) inhibits the activity of ATP:PFK; this inhibition is relieved by Pi (Plaxton, 1996). In addition, ATP:PFK activity may be inhibited by low pH. Low O_2 or high CO_2 reduce cytoplasmic and vacuolar pH and ATP levels in lettuce (Siriphanich and Kader, 1986), which, in turn, affected the activity of ATP:PFK and PPi: PFK (Hess et al., 1993).

Pyruvate kinase (PK, ATP:pyruvate 2-*O*-phosphotransferase, EC2.7.1.40), is a regulatory enzyme of glycolysis that catalyzes the irreversible synthesis of pyruvate (PYR) and ATP from PEP and ADP (Knowles et al., 1998). This step is now recognized as the

primary control site of glycolytic flux to pyruvate in plants (Beaudry et al., 1989 and 1987; Plaxton, 1996). This enzyme is localized in the cytoplasm (PK_c) and in the plastids (PK_p), where they differ by immunological and kinetic properties (Blakeley and Dennis, 1993). PK_c is suggested to play a key role in C3 plants regulation of glycolytic flux, thereby affecting mitochondrial respiration and carbon skeleton generation for anabolic reactions (Knowles et al., 1998; Plaxton, 1996). Studies on PK_c and PK_p at the mRNA and protein levels in developing tobacco seeds demonstrated that the expression of these isozymes may be controlled by independent transcriptional and posttranscriptional mechanisms, and PK_p has a much greater rate of turnover than PK_c (Blakeley and Dennis, 1993). However, PK_c activity also may be modulated by fine control mechanisms (Plaxton, 1996). For example, high levels of ATP inhibit PK allosterically in higher plants, by decreasing the affinity of the enzyme for its substrate PEP (Plaxton, 1996; Turner and Turner, 1980). PK is also inhibited by acetyl-CoA and by long-chain fatty acids (Plaxton, 1996).

High levels of CO_2 can either slow or elevate respiratory rates depending on the commodities and the concentration of CO_2 employed (Beaudry, 1993; Cameron et al., 1994; Gran and Beaudry, 1993; Kerbel et al., 1988). Lower O_2 levels also reduce the respiration rate of fresh fruits and vegetables. Storage of fruits and vegetables in less than 15 kPa CO_2 can cause changes in the activity of respiratory metabolism and may have an uncoupling effect on oxidative phosphorylation (Lange and Kader, 1997a). Air plus 10 kPa CO_2 inhibits glycolysis in intact pear fruit, probably due to CO_2 effects on the glycolytic enzymes, especially for ATP:PFK and PPi:PFK (Kerbel et al., 1988). According to Baxter and Waters (1991), asparagus stored under controlled atmosphere

(CA) of 5 kPa O_2 and 10 kPa CO_2 retained more sugars than spears stored in air at room temperature. The greater retention of sugars for CA-stored spears compared to air-stored spears implicates a general impairment of glycolytic reactions by CO_2.

In asparagus spears tips, effects of O_2 and CO_2 on the pool of intermediate and the activity of glycolytic enzymes has not been studied. This effect is expected to be more pronounced for PFK and PK. The effects of low O_2/high CO_2 levels on intermediate pools and enzymes of glycolysis in asparagus is not known. The response is addressed in Chapter 4.

Fermentative metabolism. Low O_2 dramatically affects plant metabolism by impairing the oxidative phosphorylation pathway. The enzymes and cofactors for this pathway are located in the mitochondrial membrane. The oxidative phosphorylation pathway is normally responsible for the regeneration of NADH and NADPH in the form of reducing power, which is critical for glycolysis, with a concomitant production of ATP. Limited availability of O_2 as terminal electron acceptor for oxidative phosphorylation stops ATP production via this pathway and depletes the resources of NAD^+ and $NADP^+$ (Dolferus et al., 1997). Earlier views of the response to limiting levels of O_2 at the cell level were formalized in the concept of the Pasteur effect. As ATP generation by oxidative phosphorylation begins to fall off due to insufficient O_2, the energetic deficit is made up by activation of anaerobic ATP supply pathways. In some cases, this kind of O_2 sensing would mean activation of anaerobic glycolysis (since this is the only known mechanism for ATP generation without O_2 in the cell). Contrary to this point of view, as a means for sensing hypoxia, it is argued that hypoxia tolerant tissues use anaerobic

metabolism not to make up energy deficits but to sustain a reduced energy turnover state instead (Hochachka et al., 1996).

In passive MAP of fresh produce, there is a risk that oxygen levels could fall below critical levels and cause product fermentation or even favor the growth of harmful human pathogens (Cameron et al., 1994). There is considerable risk associated with MAP systems when attempts are made to reduce the O_2 concentration inside the package to low levels at low temperatures because anaerobic conditions can develop if temperature increases during subsequent storage or handling (Cameron et al., 1994). In asparagus stored at elevated temperatures, the rate of loss of carbohydrates, proteins, and amino acids are also more accentuated than at lower temperatures (Saltveit and Kasmire, 1985).

Anaerobiosis increases the rate of quality loss of asparagus during storage (Torres-Penaranda and Saltveit, 1994). Anaerobic respiration develops below a critical O_2 level of 2.3 kPa at 20 °C as shown by an increase in CO_2 production relative to aerobic respiration (Platenius, 1943). Asparagus spears exposed to anaerobiosis for 6 h at 20°C and for 4h at 2.5 °C resulted in a stimulation followed by a decline in CO_2 production. The physiological cause for that was not explained (Torres-Penaranda and Saltveit, 1994), however, it is possible that accumulated products of anaerobic respiration may be responsible for such response. Very low levels of O_2 may cause the accumulation of ethanol and acetaldehyde in fruits and vegetables (Ke and Kader, 1990). In asparagus held at 20 °C, an alcoholic aroma started to appear for O_2 levels below 2 kPa (Platenius, 1943). The effect of O_2 on the synthesis of fermentation products is further modulated by the presence of CO_2 (Shirazi et al., 1991; Thomas, 1925). In addition, the combination of low

O_2 and high CO_2 had a synergistic effect on inhibiting ATP biosynthesis (Hess et al., 1993). Lipton (1965) found that an atmosphere of 1 kPa O_2 for 7 days at 6 °C reduced soft rot, which was eliminated by 0 kPa O_2. However, 0 kPa O_2 resulted in low-O_2 injury to the spear. In avocado, anaerobic atmospheres induced the accumulation of ethanol and acetaldehyde to levels that caused off-flavor and an enhancement of some physiological disorders (Ke and Kader, 1990). Saltveit and Ballinger (1983) found that ethanol concentration in asparagus spears increased linearly for up to 4 h under N_2 or CO_2 atmosphere and exponentially with temperatures from 0 to 30 °C.

Severe stress levels of O_2 substantially reduce NADH flux to the cytochrome electron transport system and oxidative phosphorylation is greatly reduced. Pyruvate dehydrogenase activity is reduced and pyruvate flux to the TCA cycle decreases. Decreased carbon flux through the TCA cycle directs PYR to the fermentation pathway and cause the accumulation of acetaldehyde and ethanol (Ke et al., 1993). Some plant species delay or avoid accumulation of ethanol by diverting glycolytic intermediates to alternate end products such as lactate and malate as a result of limited O_2 supply (Kennedy et al., 1992). According to Kennedy et al., (1992), lactate dehydrogenase (LDH, L-(+)-lactate:NAD+ oxidoreductase, EC1.1.1.27), pyruvate decarboxylase (PDC, 2-oxoacid carboxylase, EC 4.1.1.17), and alcohol dehydrogenase (ADH, alcohol:NAD oxidoreductase, EC 1.1.1.1) are among the primary enzymes involved in anaerobic metabolism in plants.

LDH and PDC are generally present in very low amount in aerobic tissues and often suggested to be limiting (Germain et al., 1997). LDH catalyzes the formation of

lactate from pyruvate, is present as multiple isoenzymes, and is anaerobically induced in several species. Lactate fermentation generally precedes ethanol production as O_2 levels become increasingly hypoxic (Davis, 1980). The relative rate of synthesis of lactate versus ethanol, however, depends upon the cytoplasmic pH (Davies, 1980). Low O_2 and/or high CO_2 stresses may change intracellular pH (Hess et al., 1993). At the onset of anaerobiosis, PYR is initially converted to lactate, resulting in a drop in cytoplasmic pH. The drop in pH reduces LDH activity, but activates PDC; subsequently, ethanol is predominantly synthesized by ADH (Davies, 1980; Germain et al., 1997). Based on this scenario, Davis, (1980) suggested that LDH and PDC act as a metabolic pH-stat for regulating fermentative metabolism.

PDC catalyzes the decarboxylation of pyruvate, yielding CO_2 and acetaldehyde, the precursor of ethanol. In maize, PDC activity may increase 5- to 9-fold during anoxia whereas PDC mRNA may be induced about 20-fold (Kelley, 1989). ADH governs ethanol formation. In response to anaerobiosis, ADH activity increases in most plants. ADH activity may represent a flood tolerance strategy in some species. For instance, ADH activity seems to be essential for survival during short periods of flooding in maize (Drew, 1990), and may also be energetically advantageous for survival under O_2 and/or CO_2 stresses.

Asparagus responses to very low O_2/ high CO_2 concentrations by inducing the fermentative pathway. This may lead to a decrease in intracellular pH and ATP levels and the accumulation of ethanol. Lactate accumulation is an alternative pathway that can occur in asparagus. The regulation of ethanol and/or lactate fermentation in asparagus in

response to low O_2/high CO_2 stresses is not known. This response is addressed in Chapter 3.

Phosphate and adenylate metabolism. Plant tissues use molecular oxygen for a number of biosynthetic or degradative processes (Kennedy et al., 1992), the most important one being mitochondrial respiration (Ricard et al., 1994). Phosphate metabolism is a vital component in energy transfer during the reduction of O_2. Phosphate is also used in metabolic regulation and is an important constituent of macromolecules such as phospholipids and nucleic acids (Duff et al., 1989). The growth and development of plants is particularly dependent upon the availability of Pi, and the effects of environmental Pi deficiency on the rates of photosynthesis and photoassimilate partitioning in plants has been studied (Duff et al., 1989; Goldschmidt and Huber, 1982; Preiss and Sivak, 1996). Starch synthesis in leaf cells is mainly controlled by the ADPG-pyrophosphorylase (EC 2.7.7.27), which catalyzes the interconversion of glucose-6-phosphate to ADP-glucose. Its activity is largely regulated by the intracellular Pi concentration and triose phosphate availability (Preiss and Sivak, 1996).

Phosphate starvation is suggested to induce bypass of adenylate- and phosphate-dependent glycolytic enzymes when intracellular pools of ATP, ADP, and Pi are depleted (Duff, et al., 1989). Pi content also seems to be altered by sucrose levels. For instance, in sycamore cells, when sucrose levels dropped to critical levels in the cell, the levels of intracellular Pi increased (Rébeillé et al., 1985). Pi content is also affected by O_2 and CO_2 partial pressures. Its content increases in avocado tissues in response to elevated CO_2 levels (Lange and Kader, 1997b) and in minimally processed carrots treated with 0.5 and

2.0 kPa O_2, at 5 and 15 °C, when compared to air (Kato-Nogushi and Watada, 1996b).

Pyrophosphate (PPi) is thought to be controlled in the cytoplasm by PPi:PFK, which produces PPi during gluconeogenic carbon flux. PPi concentration may enhance or regulate sucrose breakdown via sucrose synthase. PPi:PFK is also able to maintain PPi levels in the cytoplasm according to its supply and demand (Dancer and Ap Rees, 1989). PPi, therefore, serves as energy source for glycolysis (Carnal and Black, 1979). PPi is also a by-product of a host of reactions involved in macromolecule biosynthesis. One dogma of cellular bioenergetics is that the anhydride bond of PPi is never utilized and that PPi produced in biosynthesis is always removed by the hydrolytic action of an inorganic alkaline pyrophosphatase, thereby providing a thermodynamic "pull" for biosynthetic processes (Plaxton, 1996). PPi-dependent processes may be a crucial mechanism for metabolic adaptation of plants to environmental stresses that reduce NTP availability via increased activity of sucrose synthase (Guglielminetti et al., 1995; Huber and Akazawa, 1986), PPi;PFK (Duff et al., 1989), and the tonoplast H^+-PPiase (Plaxton, 1996).

Energy transduction and energy storage, involving the adenine nucleotides (ATP, ADP, AMP), are fundamental features of metabolism. Adenine nucleotides form an extensive systems of allosteric regulators that operate on a large number of reactions to adjust the rate of ATP synthesis to its utilization (Atkinson, 1977; Pradet and Raymond, 1983). Adenine nucleotides are maintained in equilibrium by the enzyme adenylate kinase (EC 2.7.4.3) which catalyzes the reaction,

$$ATP + AMP \leftrightarrow 2\ ADP \quad [4].$$

Therefore, a decrease in the concentration of ATP will occur following an increase in that

of AMP, and vice versa. The concept of 'adenylate energy charge' (AEC), which represents the relative saturation of the adenylate pool in phosphoanhydride bonds, was introduced by Atkinson (1977), which is expressed as:

$$AEC = ([ATP] + 0.5 \cdot [ADP]) \cdot ([ATP] + [ADP] + [AMP])^{-1} \quad [5].$$

AEC can theoretically vary between 0 and 1. Usually, the AEC of actively metabolizing plant tissues varies from 0.8 to 0.85 (Pradet and Raymond, 1983). Regulatory enzymes that occur in pathways in which ATP is utilized respond to changes in energy charge in the general way shown by curve 2 of Fig. 1.3. Conversely, regulatory enzymes that participate in pathways in which ATP is regenerated respond to the pattern shown in curve 1 (Plaxton, 1990). According to Fig. 1.3, in a healthy cell, any tendency for AEC to fall would be prevented through ATP-regenerating pathways (curve 1) and by the decrease in flux through ATP-utilizing pathways (curve 2). Plant tissue under anoxia generally results in a large decrease in AEC (Saglio et al., 1980). Under hypoxia, tissue metabolic activity is limited by the availability of oxygen, and it appears that the level of AEC is clearly correlated with metabolic activity (Pradet and Raymond, 1983).

The ATP/ADP ratio is a fundamental regulatory and regulated value, which is sensed by the enzymes and is related to the thermodynamic state of the adenine nucleotide pools (Pradet and Raymond, 1983). For instance, low ATP/ADP ratios of anoxic tissues resulted in strong inhibition of HK (Renz and Stitt, 1993), and, thus, glycolytic flux. The levels of ADP in the cell are substrates for oxidative phosphorylation in the mitochondria, hence, the absolute cytoplasmic concentration of ADP available for uptake into that organelle controls the rate of respiration (Rébeillé et al., 1985). In anoxic maize roots,

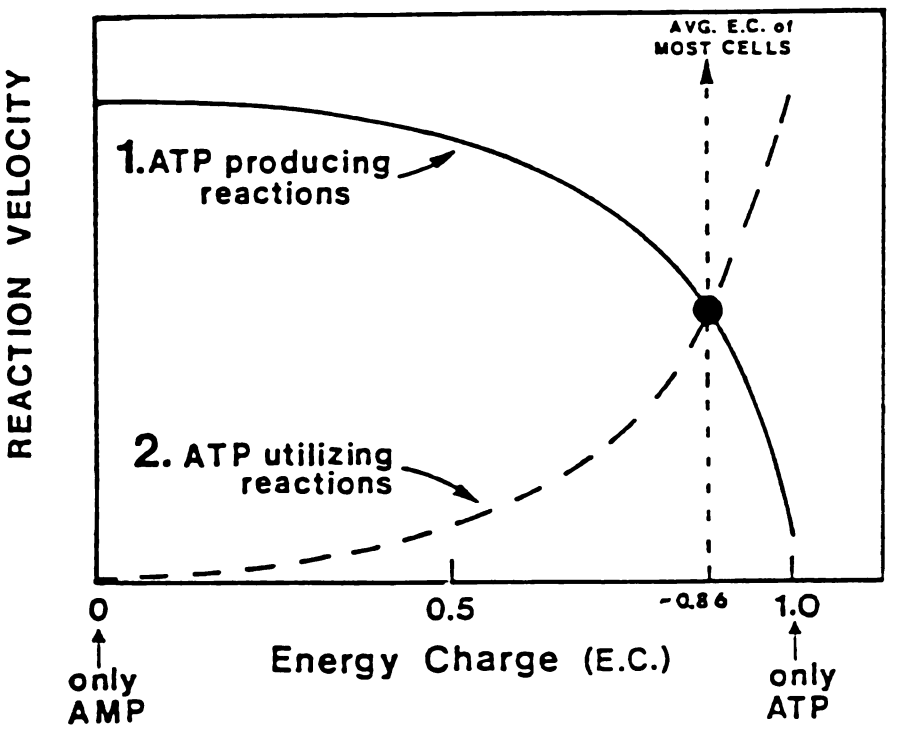

Figure 1.3. Responses to the cellular energy charge of regulatory enzymes in metabolic pathways in which ATP is produced (curve 1) and in which it is utilized (curve 2). From Plaxton, (1990).

10 h of anoxia resulted in increased ADP with a concomitant decrease in ATP content (Bouny and Saglio, 1996).

In asparagus spear tips, the pool of phosphate and adenylates, and AEC may be affected when spears are stored under low O_2/ high CO_2. The effects of lower O_2 /high CO_2 on phosphates, adenylates and AEC is unknown for asparagus and is addressed in Chapter 5.

Cytokinin and Carbon Allocation

Senescence in mature plant parts can be induced or enhanced by the influence of growing organs (e.g. asparagus tips), and this relationship affects the transport process. In maize leaves, the start of senescence was delayed by interrupting the transport of metabolites to sink tissues (Müller and Leopold, 1966b). In asparagus, rapid depletion of sucrose in the tip (King et al., 1990) with concomitant acropetal decline of soluble sugar (Silva et al., 1998), suggests that the tip may have a regulatory influence on the after harvest physiology of the remaining spear in acting as a sink that "scavenges" for metabolites translocated from lower parts of the spear (Saltveit and Kasmire, 1985).

It has been hypothesized that allocation of carbon is mediated by cytokinin and sucrose (Van der Werf and Nagel, 1996). Exogenous application of cytokinins to plant tissues results in various responses including a delay in senescence, increased chlorophyll production, stimulation of chloroplast development, a decline in chlorophyll degradation, the promotion of protein and nucleic acid synthesis, and the mobilization of nutrients into the cytokinin-treated area (Taiz and Zeiger, 1991). Cytokinins are also involved in the maintenance of photosynthetic activity (Wingler et al., 1998) through effects on

chlorophyll degradation and inhibition of the loss of photosynthetic proteins (Badenoch-Jones et al., 1996). Exogenously applied cytokinin promotes production of, and increased activity of, ribulose bisphosphate carboxylase (Clarke et al., 1994). Cytokinin was also effective in delaying chlorophyll degradation and decreasing rate of respiration in harvested broccoli (Clarke et al., 1994; Rushing, 1990). Senescence has been delayed in transgenic plants by expression of a bacterial gene encoding isopentenyl transferase, the enzyme catalyzing the first step of cytokinin synthesis (Gan and Amasino, 1995). However, it has been suggested that cytokinin, in addition to delaying senescence, could block some responses to changing sugar levels (Jang et al., 1997). Cytokinin was effective in stimulating a mass-flow in the phloem, attracting substances from untreated tissue and directing them toward the site of application (Müller and Leopold, 1966a and b). The interaction of a supplier region and a mobilizer region involving a redistribution of material was termed 'cytokinin-induced directed transport'.

Thus, the evaluation of the physiological changes and mechanisms that may regulate carbon flux and the rate of utilization of sugars throughout the spears may contribute much to understanding the deteriorative processes that accompany postharvest of asparagus. It is possible that cytokinin application in spear tips would enhance carbon flux in its direction. This would minimize the rate of sucrose degradation in the tip and improve the quality of asparagus spears stored at 1°C. This response is addressed in Chapter 6.

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CHAPTER 2

RESPIRATORY AND FERMENTATIVE METABOLISMS IN ASPARAGUS SPEAR TIPS UNDER LOW O_2/HIGH CO_2 ATMOSPHERES

INTRODUCTION

Asparagus spears senesce rapidly after harvest. The process of senescence is associated with many physiological and biochemical changes. Among these changes, sugar depletion is notable. The sugar content of asparagus spears declines rapidly following harvest. The decline in sugar is most marked in the spear tip, a meristematic tissue that is recognized to be a very active sink (Hurst et al., 1993b). The predominant sugars in asparagus at harvest, sucrose (Irving and Hurst, 1993), is translocated from the crown during the period of rapid spear growth (Robb, 1984). Sucrose is utilized to provide energy for global metabolic processes and to a significant extent, is the source of carbon skeletons for cell wall formation. The rapid use of imported sucrose sustains a sucrose concentration gradient between the sink cell and the phloem tissue (Ho, 1988).

It has been suggested that sucrose rapid decline in the spear tip triggers or signals deterioration in the whole spear (King et al., 1993). Thus, knowledge of the processes that govern the sugar content may be important in maintaining the quality of asparagus postharvest. Importantly, understanding the pattern of sucrose decline in the tip could help elucidate a means to control its loss, and, subsequently, control spear deterioration. Ultimately, it may be possible to extend the shelflife of asparagus spear.

Exposing harvested horticultural crops to low levels of O_2 with or without high levels of CO_2 has been shown to affect many causes of deterioration and influence crops

quality and increase storage life (Kader, 1986). Elevated CO_2 atmospheres seem to have a particular effect on sugar content in asparagus (Baxter and Waters, 1991). Treating asparagus with elevated CO_2 for a limited period also has potential as a quarantine treatment for insect control (Corrigan and Carpenter, 1993). Different commodities exhibit widely different thresholds to O_2 and CO_2 that are both temperature- and concentration-dependent (Saltveit, 1993). However, some undesirable physiological changes may also occur in plant tissues under stress levels of low O_2 and high CO_2. The potential benefit of using low O_2/high CO_2 atmospheres in storage depends on several variables. High levels of CO_2 can either reduce or elevate respiration rates depending on the commodity and the concentration of CO_2 utilized (Beaudry, 1993; Kerbel et al., 1988). Elevation of the respiratory quotient (RQ) can also occur in response to elevated CO_2 levels (Thomas, 1925). The increase in RQ is accompanied by enhanced synthesis of ethanol and acetaldehyde (Thomas, 1925). The effects of CO_2 on the synthesis of fermentation products is further modulated by the presence of O_2 (Beaudry, 1993; Shirazi et al., 1991; Thomas, 1925).

Higher plants have an absolute requirement for oxygen as a terminal electron acceptor during respiration (Kennedy et al., 1992). Plant responses to low O_2 and high CO_2 levels include induction of fermentative metabolism and a decrease in ATP content (Ke et al., 1994; Saglio et al., 1980) (Fig. 2.1). One pathway of fermentative metabolism results in accumulation of acetaldehyde, catalyzed by the enzyme pyruvate decarboxylase (PDC, EC 4.1.1.1) and ethanol, catalyzed by the enzyme alcohol dehydrogenase (ADH, EC 1.1.1.1). Some plant species delay or avoid accumulation of ethanol by diverting

pyruvate to lactate via a reaction catalyzed by the enzyme lactate dehydrogenase (LDH, EC 1.1.1.27) (Guglielminetti et al., 1997). It is believed that the primary function of fermentative metabolism is to use NADH and pyruvate when electron transport and oxidative phosphorylation are inhibited so that NAD is recycled and glycolysis can proceed. This allows for continued carbon flux and the production of some ATP, which will permit the plant tissue to survive temporarily under O_2-deficient conditions.

Modification of storage atmosphere has been long used as a means to develop a better understanding of the biochemical and physiological processes in crops (Kays, 1991). Although intensive research has been done on anaerobic metabolism in maize, rice, pears, avocado (Davis, 1980; Ke et al., 1995; Kennedy et al., 1992), the mechanism for regulation of low O_2 and high CO_2 on ethanolic fermentation in asparagus has not been studied (Baxter and Waters, 1991; Hurst et al., 1997; King et al., 1986; Lipton, 1965). In this research, the effects of CO_2 (0, 5, 10, and 20 kPa) combined O_2 partial pressures ranging from 0.1 to 16 kPa (1% O_2 = 1.013 kPa O_2 at 1 atm) were evaluated. The purpose of this work was to investigate the influence of CO_2 and O_2 partial pressures on sugar utilization, respiratory activity, fermentative metabolism, and quality in asparagus spears stored at 1 °C.

MATERIAL AND METHODS

Plant Material. Spears (*Asparagus officinalis* L. cv. Jersey Giant) were harvested in May from the Michigan State University Horticultural Research Center between 6:00 and 9:00 a.m. Spears were harvested at a length of 240 mm, trimmed to 180mm, graded,

placed on ice and held at 0 °C. Only straight undamaged spears with closed bracts and no obvious disease symptoms were used. A set of spears were used for determination of sugar and lactate contents and enzyme activities at harvest. These spears were trimmed and frozen in liquid N_2 in the field. These and all subsequent samples were stored at -80 °C until analysis. Three entirely separate experiments were conducted in three consecutive asparagus seasons (1995, 1996, 1997) and the obtained data combined. Asparagus spears were held at 1 °C throughout this study.

Steady State O_2 and CO_2 and Respiration Rates. The levels of O_2 generated ranged from approximately 0.16 to 16 kPa for each of four different treatment levels of CO_2 (0, 5, 10, and 20 kPa). Spears were enclosed within a package composed of low density polyethylene (LDPE) film and having a surface area of 462 cm^2. The packages for the 5, 10, 20 kPa CO_2 treatments were enclosed in 1.95-L glass jars using the method of Beaudry (1993). Each package was equipped with a gas sampling septum (Boylan-Pett, 1986). For packages in jars, the septum was attached to the base of a 1.3 cm internal diameter (I.D.) x 2.7 cm long glass tube using silicone sealant. The glass tube was inserted through the jar lid and attached to the surface of the enclosed package (Fig. 2.2). Chamber lids were also fitted with inlet and outlet ports for purging with gas mixtures. Chambers were continually purged with a mixture of air, CO_2, and N_2 gases at 30 $mL \cdot min^{-1}$ to generate target atmospheres within the enclosed packages. Packages were designed to achieve target O_2 levels based on measured permeabilities of the LDPE films to O_2 and CO_2, and by altering film thickness, and the total spear mass within the packages. Package gas composition was determined daily by withdrawing a 100-μL gas sample from the

package with a 500-μL gas-tight syringe (Hamilton Co. Reno, Nevada) and analyzing for O_2 and CO_2 using O_2 (Servomex series 1100 with a paramagnetic detection cell) and CO_2 (ADC 225-MK3 analytical infrared gas analyzer) analyzers connected in series with N_2 as a carrier gas (flow rate=100 $mL \cdot min^{-1}$). Gas concentrations were converted to partial pressures. The gas composition of individual packages was monitored until steady state conditions were reached (approximately six days). O_2 and CO_2 gradients across the film were used to calculate steady state diffusion for both gases through the film using previously obtained permeability data (Lakakul, 1994). Flux data were used to calculate the rates of O_2 uptake and CO_2 production (Cameron et al., 1994).

Measured O_2 and CO_2 gradients across the film were used to calculate steady state diffusion rates for both gases through the film. Respiration rates were calculated from the gas diffusion rates by assuming diffusion rates though the package were equal to the O_2 and CO_2 fluxes in and out of the fruit. The data were combined to ascertain the rates of respiration using the following formulae:

$$RR_{O_2} = \frac{(P_{O_2} A/x)([O_2]_{chmbr} - [O_2]_{pkg})}{W} \quad [1]$$

$$RR_{CO_2} = \frac{(P_{CO_2} A/x)([CO_2]_{pkg} - [CO_2]_{chmbr})}{W} \quad [2]$$

where RR_{O_2} and RR_{CO_2} are the rates of O_2 uptake and CO_2 production ($mmol \cdot kg^{-1} \cdot hr^{-1}$), respectively; P_{O_2} and P_{CO_2} are measured O_2 and CO_2 permeability coefficients

(mmol·cm·cm^{-2}hr^{-1}·kPa^{-1}), respectively, for LDPE at the storage temperature; A is film area (cm^2); x is thickness (cm); $[O_2]_{chmbr}$ and $[O_2]_{pkg}$ are chamber and package partial pressures of O_2 (kPa), respectively; $[CO_2]_{pkg}$ and $[CO_2]_{chmbr}$ are package and chamber CO_2 partial pressure (kPa), respectively; and W is asparagus spear weight (kg). The RQ was calculated as RR_{CO2} divided by RR_{O2}. Non-oxidative CO_2 (CO_2 not used in oxidative reactions) was calculated by subtracting RR_{CO2} from RR_{O2} (mmol·kg^{-1}·hr^{-1}). Steady state levels were achieved on day 6. Tissue samples (apical 45 mm section) were taken on day 6 and stored at -80 °C for soluble sugar, lactate and fermentative enzyme determinations. Spears were combined into three replication jars for each parameter evaluated.

Ethanol and acetaldehyde determinations. Headspace ethanol and acetaldehyde were measured by a gas chromatograph equipped with a flame ionization detector (Series 400, Hach Carle Co., Series 400, Loveland, CO). A sample of 1 mL was withdrawn from each package using a gas-tight syringe (Hamilton Co., Reno, Nevada). The chromatograph column (4 m long x 3 mm I.D.), was packed with 10% DEGS-PS, 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) and was maintained at 110 °C. The carrier gas was He and the flow rate was 40 mL·min^{-1}. The flow rate of H_2 and air were 25 and 200 mL·min^{-1}, respectively. Standard curves were prepared for ethanol and acetaldehyde with analytical grade products (Sigma Chemical Co., St. Louis, MO) and using the method of Buttery et al. (1969).

Sugar Determinations. Sugars were extracted from approximately 3.0 g of frozen tip tissues by adding 3.5 mL of 80% ethanol and grinding with a tissue homogenizer (Ultra Turrex Model, Tekmar Co., Cincinnati, OH), and held for 15 min at room temperature.

The mixture was centrifuged (Model RG-5C, Sorval Instruments, Dupont Co., Newton, CT) at 2,000 *g* for 5 min and the supernatant decanted into a capped 50 mL centrifuge tube. The pellet was resuspended and extracted two additional times. The extract was then washed out with 5 mL of water. To remove traces of chlorophyll, 5 mL of chloroform was added to the combined 15.5 mL of ethanol/water mixture. The tubes were capped, shaken vigorously and then centrifuged at 1,000 *g* for 3 min. The upper, clear aqueous phase containing the soluble sugars were transferred to a 15 mL test tube and evaporated to dryness using a speedvac equipped with a refrigerated condensation trap (RT4104) maintained at -103 °C (SC200 Savant Speedvac, Savant Instruments, Inc., Farmingdale, NY). Fructose, glucose, and sucrose were separated and quantified by a gas chromatograph (Hewlett Packard Model HP5890 Series II, Hewlett Packard Co., Palo Alto, CA) fitted with an FID and a capillary colunm (30 m long x 250 μm i.d.) having a 0.25 μm film thickness DB1702 capillary column.

Lactate extraction. Approximately 5 g of frozen tissue per replicate was transferred to liquid N_2 in a precooled mortar and ground with a pestle to a fine powder. Ten mL of ice-cooled 0.8 N (v/v) $HClO_4$ were added to the powder, which was extracted for 30 min at 0 °C with occasional shaking, followed by centrifugation at 30,000 *g* for 30 min at 0 °C. Supernatant pH was adjusted to 7.0 to 7.2 with 5 N K_2CO_3, allowed to sit on ice for 10 min, and clarified by centrifugation at 30,000 *g* for 10 min. The resulting supernatant was used for lactate analysis. To estimate losses of this metabolite during extraction, measured amounts of pure lactate roughly equal to the amounts present in the tissue, were added to the extracting medium. Recovery was 91%. The recovery assay

was replicated three times.

Lactate assay. Lactate was assayed spectrophotometrically (Hitachi, Model U3000, Hitachi Inc., Tokyo, Japan) at 25 °C by measuring changes in the absorbance of pyridine nucleotides at 340 nm due to oxidation/reduction following adaptations from procedures by Noll (1984) and Scott et al., (1995), using a double-beam spectrophotometer (Hitachi, Model U3000, Hitachi Inc., Tokio, Japan), equipped with enzyme module. The determination of lactate was assayed in a final volume of 1 mL containing 40 mM glycylglycine/L-glutamate buffer (pH 9.0), 0.5 mM NAD, 2 units L-alanine: 2-oxyglutarate aminotransferase, and 2 units lactic dehydrogenase (LDH).

Extraction of Pyruvate Decarboxylase (PDC), Alcohol Dehydrogenase(ADH) and LDH. Approximately 3 g of frozen tip tissue was ground as described previously and mixed with a 10 mL ice-cold 100 mM 2-(N-morpholina) ethane-sulfonic acid (MES) buffer (pH 6.5), containing 2 mM dithiothreitol, 50 mg/g tissue of insoluble (40,000 mw) PVPP, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 10 % (v/v) glycerol. The mixture was centrifuged at 30,000 *g* for 10 min. The supernatant was used as enzyme extract for the immediately measurement of PDC, ADH, and LDH activities (Ke et al., 1994).

Enzyme Assays. Assays for enzyme activities were run immediately after extraction in a final volume of 1 mL based in modifications from Ke et al. (1994). The assay cocktails were as follows: Alcohol dehydrogenase: 80 mM MES (pH 6.5), 0.2 mM NADH, 50 µL of the enzyme extract, and 10 mM of acetaldehyde (Ashihara et al., 1989). Lactate dehydrogenase: 74 mM MES (pH 6.5), 0.2 mM NADH, 4 mM pyruvate, 3 mM of 4-methylpyrazol, 3 mM KCN, and 100 µL of extract (Ke et al., 1994). Pyruvate

decarboxylase: 60 mM MES (pH 6.5), 0.5 mM thiamine pyrophosphate, 5 mM $MgCl_2$, 0.5 mM NADH, 15 units ADH, 50 µL of enzyme extract, and 10 mM pyruvate (start reaction) (Nanos et al., 1992). The measurement of the oxidation of NADH for ADH and LDH was followed at 30 °C by recording the decrease in absorbance at 340 nm as previously described.

Sensory quality. Sensory quality was rated for the whole spears upon opening each LDPE bags and exposing the spear to room temperature for 12 hr after the steady state O_2 was reached (following 6 days of storage). Each treatment replication was rated independently by the subjective evaluation of four observers using the following scale adapted from Hurst et al. (1993a):

9= fresh like;

7= slight browning of tips and scales, slight flaccidity and/or very slight wrinkle on stems, absence of strange flavors;

5= moderate browning of tips and/or stem bracts browning, more pronounced wrinkle, loss of pink blush at the base, slight ethanol odor;

3= extensive browning and pronounced flaccidity of tips and stems bracts (feathering), wrinkle and wilting of stems, moderate fermentative odor;

1= extensive browning and flaccidity of tip and stems bracts, complete loss of pink blush at the base, extensive wrinkle and wilting stems and base, brown spot in stems when exposed to air, strong fermentative odor.

Asparagus rating of 6 was considered unacceptable for sale although still edible.

RESULTS

Respiration rate. The rate of O_2 uptake declined with decreasing O_2 partial pressures with the rate of decline being most rapid below 2 kPa O_2 (Fig. 2.3). O_2 uptake was apparently unaffected by CO_2 partial pressures. CO_2 production behaved similarly to O_2 uptake with respect to O_2 partial pressures. However, CO_2 production was enhanced by the 20 kPa CO_2 treatment. Above 2 kPa O_2, the RQ was approximately 1 for 0, 5, and 10 kPa CO_2 treatment. The RQ averaged 1.2 for the 20 kPa CO_2 treatment in this O_2 range. Below 2 kPa O_2, the RQ increased markedly as CO_2 levels increased. Non-oxidative CO_2 production tended to increase as CO_2 levels increased. Non-oxidative CO_2 production also tended to increase as O_2 levels declined, with the most rapid increase occurring below 2 kPa O_2 (Fig. 2.4), following the same pattern of RQ. Interestingly, it appeared that the profile of the non-oxidative CO_2 production peaked at approximately 1 kPa O_2 for all four CO_2 treatments, declining sharply as the O_2 partial pressure dropped below 1 kPa.

Sugar Metabolism. There was a drop in glucose, fructose and sucrose levels in spear tips following harvest in the time required to achieve steady state O_2 conditions. Sucrose was the prevalent sugar at harvest and the one that declined most markedly after 6 d of storage at 1 °C (Fig. 2.5). For spear stored at near-ambient conditions (> 16 kPa O_2, 0 kPa CO_2) the reduction in sucrose, glucose and fructose was 85, 75 and 45% of at-harvest contents, respectively. The decline in sucrose was greatest when asparagus spear tips were exposed to 20 kPa CO_2 although the reduction in sucrose for the 0 kPa CO_2 treatment was nearly as extensive (Fig. 2.5). The greatest retention of sucrose occurred

for spears exposed to 5 and 10 kPa CO_2. At O_2 levels greater than 2 kPa, fructose and glucose tended to be similar across the O_2 range and largely unaffected by CO_2. Below approximately 2 kPa O_2, fructose tended to accumulate. For the 20 kPa CO_2 treatment, low O_2 further decreased sucrose content.

Fermentative Metabolism. Acetaldehyde and ethanol accumulation in the package headspace were well correlated and were also clearly dependent on both O_2 and CO_2 levels (Fig 2.6). Acetaldehyde and ethanol levels increased as O_2 declined, increasing most rapidly below 1 kPa. CO_2 tended to enhance the accumulation of both fermentation products at O_2 levels below 1 kPa. Headspace acetaldehyde levels were approximately 1/3 those of ethanol (Fig. 2.6). The fermentation threshold, when assessed as the O_2 partial pressure which causes a 20% increase in the RQ relative to the aerobic RQ, was approximately 1 kPa O_2 for all CO_2 treatments. Based on the RQ and non-oxidative CO_2, however, the lower O_2 limit for asparagus could be estimated to be approximately 1, 1.5, 2, and 3 kPa O_2 for 0, 5, 10 and 20 kPa CO_2 treatments, respectively.

The lactate content increased from around 50 nmol$\cdot g^{-1}$ on a fresh mass basis at harvest to 200 nmol$\cdot g^{-1}$ fresh mass at O_2 partial pressures below 1 kPa (Fig. 2.7). CO_2 caused lactate to accumulate at O_2 partial pressures greater than 4 kPa. The LDH activity declined with storage for spears stored in near-ambient atmosphere (Fig. 2.8). There was an increase in activity as the CO_2 partial pressure increased. LDH activity declined as O_2 declined to 2 kPa for the CO_2-treated spears. As O_2 declined below 2 kPa, LDH activity increased to a maximum at 1 kPa O_2 and declined again as the O_2

partial pressure dropped below 0.5 kPa O_2.

ADH activity declined with storage for spears in near-ambient atmosphere (Fig. 2.9). As the CO_2 partial pressures increased, ADH activity increased. Activity declined as the O_2 partial pressure decline to 4 kPa for the 10 and 20 kPa treatments. ADH activities were similar for all CO_2 treatments between 4 and 1 kPa O_2 and increased as the O_2 partial pressure declined below 1 kPa.

PDC activity exhibited much the same pattern as LDH, increasing as CO_2 increased for O_2 levels greater than 4 kPa and having a peak in the activity between 0.5 and 2 kPa O_2 (Fig. 2.10). PDH activity enhancement around that O_2 range, however, was prevalent for 20 kPa CO_2 treatment. ADH activity was approximately 3-fold higher than PDC and 10-fold higher than LDH (Figs.2.8, 2.9, and 2.10). In addition, in contrast to PDC and LDH, ADH activity increased markedly below 1 kPa O_2 without showing subsequent decrease, notedly for 20 kPaCO_2.

Sensory quality. From the results of this research at 1 °C, when spears reached the steady state at 6 d storage, the sensorial quality was best as O_2 levels increased and CO_2 levels decreased (Fig. 2.11). The lowest level of sensorial quality was detected at 20 kPa CO_2, which showed marked alteration of visual apparency and strong off-flavor, probably due to accumulation of fermentative products. The best quality acceptance was found for 5 kPa CO_2 treatment, which crossed the sensorial threshold (grade 6) at approximately 5 kPa O_2. At 0 kPa CO_2, the visual quality was acceptable above 6 kPa O_2.

DISCUSSION

The imposition of O_2 limitation, which results in inhibition of oxidative phosphorylation causes a rapid decline in energy metabolism is asparagus spears, and may result in death of the tissues if anoxia exists for a prolonged period of time. This may be occurred in asparagus spear tips for O_2 below 1 kPa, when O_2 uptake and CO_2 production markedly decreased. This energy drop may be correlated with a decline in respiratory substrates.

Genes upregulated by carbohydrate availability in plants include many that would enhance sucrose utilization throughout storage, respiration, and biosynthetic processes. Sugar-regulated genes carries information on carbohydrate status for coordinating changes in resource utilization and allocation (Koch, 1996), which may be reflected in metabolite pools and enzyme activities. In tissues, sucrose serves as the source of organic carbon for building structural elements, is used as a metabolic fuel, or it may accumulate (Avigad, 1982). In asparagus, sucrose is thought to be translocated from the roots of growing spears (Hurst et al., 1993b) and from lower segments (Salveit and Kasmire, 1985) throughout the spear toward the tip, where it is subsequently metabolized to glucose and fructose. In asparagus spear tips, the relatively lower rate of metabolization of fructose compared to sucrose and glucose may be due to limited availability of UTP, an essential cofactor for fructose metabolism (Irving and Hurst, 1993). Furthermore, in plants, cleavage of the glycosidic bond in sucrose occurs by either sucrose synthase (EC 2.4,1,13) or invertase (EC 3.2.1.26). Sucrose synthase requires UDP as a co-substrate and produce fructose and UDP-glucose, which may go

to cell wall biosynthesis (Copeland, 1990) or may be further metabolized in several ways. In addition to its function as a glucosyl donor in glucosylation reactions, UDP-glucose may also be oxidized to UDP-Glucuronic acid for the synthesis of several structural polysaccharides and several amino acids, or may be converted by UDP-glucose pyrophosphorylase (EC 2.7.7.9) to glucose-1-P and incorporated into the glycolytic or pentose phosphate pathways (Stewart and Copeland, 1998). In contrast, invertase simply splits sucrose into glucose and fructose (Copeland, 1990). The higher sucrose content found in asparagus tips at harvest may favor higher glucose and fructose contents because sucrose may be less metabolically accessible to respiratory loss than hexose sugars (Salerno and Pontis, 1978). Here, the drop in sucrose concentration seemed to parallel a decline in respiration rate observed in asparagus spear sections (Silva et al., 1998), and in spear tips (Irving and Hurst, 1993). The more marked decline in the glucose content relative to fructose indicates that it may be preferentially utilized in glycolysis or lost to cell wall biosynthesis as sucrose is degraded by sucrose synthase to fructose and UDP-glucose. These results indicate that sucrose supplies not only demands for synthesis of structural polysaccharides and other synthetic processes but also the demand of the respiratory pathways. Maintenance of fructose levels may be explained by the recycling of fructose derived from sucrose.

Although all sugars declined when compared to at harvest, hexoses were still higher when compared to sucrose. Irving and Hurst (1993) found a similar ratio of sugars in growing asparagus spears. These improved retention of sucrose by 5 kPa CO_2 relative to 0 kPa CO_2 may result from a decline in the activity of sucrose-metabolizing

enzymes. No decline in respiration was evident for 5 kPa CO_2 treated spears, however. It is possible that the decline in respiration that occur soon after harvest was accelerated by 5 kPa CO_2 on that this CO_2 levels caused a shift in the respiratory substrate to something other than sucrose.

Increases in acetaldehyde and ethanol at less than 1 kPa O_2 are consistent with the observed increase in the RQ. The effect of CO_2 on enhanced ethanol and acetaldehyde accumulation is similar to the effect reported by Thomas (1925) as CO_2-zymasis, in which CO_2 increased the rate of fermentation. Although some ethanol may accumulate can occur without decreasing sensorial quality, accumulation of acetaldehyde and ethanol for prolonged periods may be detrimental.

In response to CO_2 exposure, an initial drop in pH may be attributed to lactate accumulation (Kennedy et al., 1992). The pH-stat hypothesis (Davis et al., 1974; Roberts et al., 1984) proposes that, in response to low O_2, lactate is initially produced from pyruvate leading to its accumulation and a corresponding fall in pH. The fall in pH activates PDC and may initiate competition between LDH and PDC for pyruvate (Davis et al., 1974). The drop in pH and shift in enzyme activity may trigger ethanolic fermentation (Kennedy et al., 1992). Thus, ethanol biosynthesis could be assigned as a mechanism to avoid acidosis, rather than to avoid anaerobiosis by itself (Kimmerer and MacDonald, 1987). Since higher plants absolutely require oxygen to sustain metabolism, most plant tissues will tolerate anoxia only for a short period of time before irreversible damage occurs (Kennedy et al., 1992). In avocado, higher CO_2 levels resulted in reduction in pH (Hess et al., 1993; Ke et al., 1995). Furthermore, an

additive effect on pH reduction was noted when high O_2 levels were combined with lower O_2 (Hess et al., 1993). At some degree of hypoxia, three factors may slow the decline in pH: transport of lactate into the vacuole, enhanced activity of PDC and ADH, or reduced activity of LDH at lower pH values (Kennedy et al., 1992). In intact tissues, the production of lactate may precede the production of ethanol. In some cases (Davis et al., 1974), as reported here, ethanol and lactate accumulated together (Figs. 2.6 and 2.7). In asparagus spears ethanol was the main product of anaerobic metabolism; however and a smaller amounts of lactate were also detected at harvest and at near-ambient conditions.

ADH gene expression and enzyme activity are upregulated during hypoxia and anoxia (Andrews et al., 1993); however, the ADH genes *adh-2* has been reported to increase during ripening (Chen and Chase, 1993). In tomatoes, *adh-1* is apparently responsible for most ADH activity under hypoxia, whereas *adh-2* is not induced (Tanksley, 1979). The results of the current work show that, besides being actives below and above 2 kPa O_2, ADH and LDH activities were also present in asparagus spear tip tissues at harvest. However, 20 kPa CO_2 caused an increase in ADH activity in asparagus tips at 16 kPa O_2. Ethanol production and accumulation was correlated with ADH activity only when O_2 levels were below the RQ breakpoint (approximately 1 kPa O_2), however. At higher O_2 partial pressures, ADH activity was not correlated with ethanol present in the tissues. This suggests strict control over carbon flux through ADH and that more than one gene is expressed at different ranges of O_2 partial pressure. Such hypoxic responses, with emphasis primarily on ethanol production by

ADH, have been extensively studied in maize (Andrews et al., 1993; Robert et al., 1984; Saglio et al., 1980), and fruits (Ke et al., 1995; Nanos et al., 1992). However, hypoxia or anoxia in asparagus has received little attention. ADH activity seems to be essential for hypoxic survival, since *adh* null mutants do not survive 24 h of anoxia (Lemke-Keyes and Sachs, 1989).

In asparagus spear tips, the drop in LDH activity around 4 kPa O_2 for all CO_2 treatments suggests that decrease in the amount of LDH may occurred. This decline in LDH activity may also suggest a switch in gene expression around 4 kPa O_2. The increase in LDH activity around 2 kPa O_2, in contrast to thr Rivoal and Hanson (1994) report, suggests that LDH in asparagus tips LDH also has a role in a long-term hypoxic response. The work here identified two features of asparagus LDH: a) extractable LDH activity changed in response to a wide range O_2 partial pressures; b) ethanol present in the tissues of asparagus spears tips does not block LDH activity, implying the presence of a positive regulatory system, as also reported by Christopher and Good (1996). CO_2 enhanced LDH activity at O_2 ranging from 5 to 16 kPa O_2, however, O_2 rather than CO_2 appeared affect most LDH activity below 2 kPa. The LDH activity behavior followed a similar pattern to that of lactate in response to O_2 partial pressures that ranged from near-ambient to near anoxic. The similarity in patterns suggests little allosteric regulation and that changes in carbon flux to lactate are largely determined by enzyme amount. The increase in LDH activity below 2 kPa O_2 suggests that, in asparagus spear tips, there was changes in gene expression under those O_2-deficient conditions.

The profile for PDC activity below 2 kPa O_2 does not close correlate with headspace acetaldehyde, since acetaldehyde increased below 1 kPa O_2 without showing subsequent decrease.

From the results of the research here and references cited, a model of action of low O_2 /high CO_2 stresses on fermentative metabolism in asparagus is presented (Fig. 2.1). Low O_2/high CO_2 partial pressures cause a substantial reduction in NADH flux through the electron transport chain (ETC). As O_2 becomes limiting or CO_2 becomes toxic, concentrations of NAD and ATP decrease while NADH levels increase. As cytoplasmic pH decreases, LDH activity is enhanced and a new ADH isozyme is induced. PDC activity is also enhanced by an increase in pyruvate concentration, which directs pyruvate to the production of acetaldehyde. Although a decrease in pH would tend to inhibit ADH, the increase in acetaldehyde and NADH concentrations and decrease in NAD drive the ADH reaction, resulting in ethanol accumulation, the major fermentation product in asparagus spears (Fig. 2.6). The increase in pyruvate and NADH concentrations, and the decreases in NAD and ATP in conjunction with a limited range of pH drop, activates LDH and direct pyruvate into lactate.

Fermentative metabolism can be regulated by two mechanisms: molecular control of the levels of PDC, ADH, and LDH, and metabolic control of the actual functions of these enzymes in plant tissue under low and/or high CO_2 stresses (Chang et al., 1983; Ke et al., 1993). According to Hochachka, et al. (1996), O_2-sensing and signal transduction control systems may be basic to the hypoxic up-regulation of genes for glycolytic enzymes, and for hypoxic suppression of Krebs cycle enzymes. In

avocado, a decrease in cytoplasmic pH substantially activated PDC but not LDH, and ADH had more affinity for NADH than that for LDH (Ke et al.,1995). In here pH was not measured; however, the results suggest that acetaldehyde produced from PDC reaction was easily converted to ethanol due to the 3-fold higher activity of ADH and predominant accumulation of ethanol over other fermentation products. Therefore, in asparagus spears, as the level of O_2 was reduced and CO_2 increased, the ethanol pathway was favored relative to the lactate pathway. Similar results have been obtained for other plant tissues such as lettuce (Siriphanich and Kader, 1986), avocado (Ke et al., 1995), and pears (Nanos et al., 1992). In addition, the results here indicate that CO_2 may have influence on the activity and amount of LDH, PDC, and ADH, as indicated by measured extractable enzyme activities as well as by the content of lactate, acetaldehyde, and ethanol.

Exposure of fruits to very low O_2 and high CO_2 concentrations at certain levels may have some effect in maintaining the quality of commodities (Saltveit, 1993). The shelflife of asparagus spears stored in air for 105 hr (approximately 4.5 d) at 0 °C was about 5 d when transferred to 20 °C (Brash et al., 1995). Recommendations for controlled atmosphere/modified atmosphere indicate that the optimum range for asparagus storage is to hold in 21 kPa O_2 and 10 to 14 kPa CO_2 at the range of 1 to 5 °C (Saltveit, 1993). The results here clearly show that combination of near-ambient O_2 level with 5 kPa CO_2 slowed down metabolism, as demonstrated by reductions in sucrose depletion, and also provided a better visual quality.

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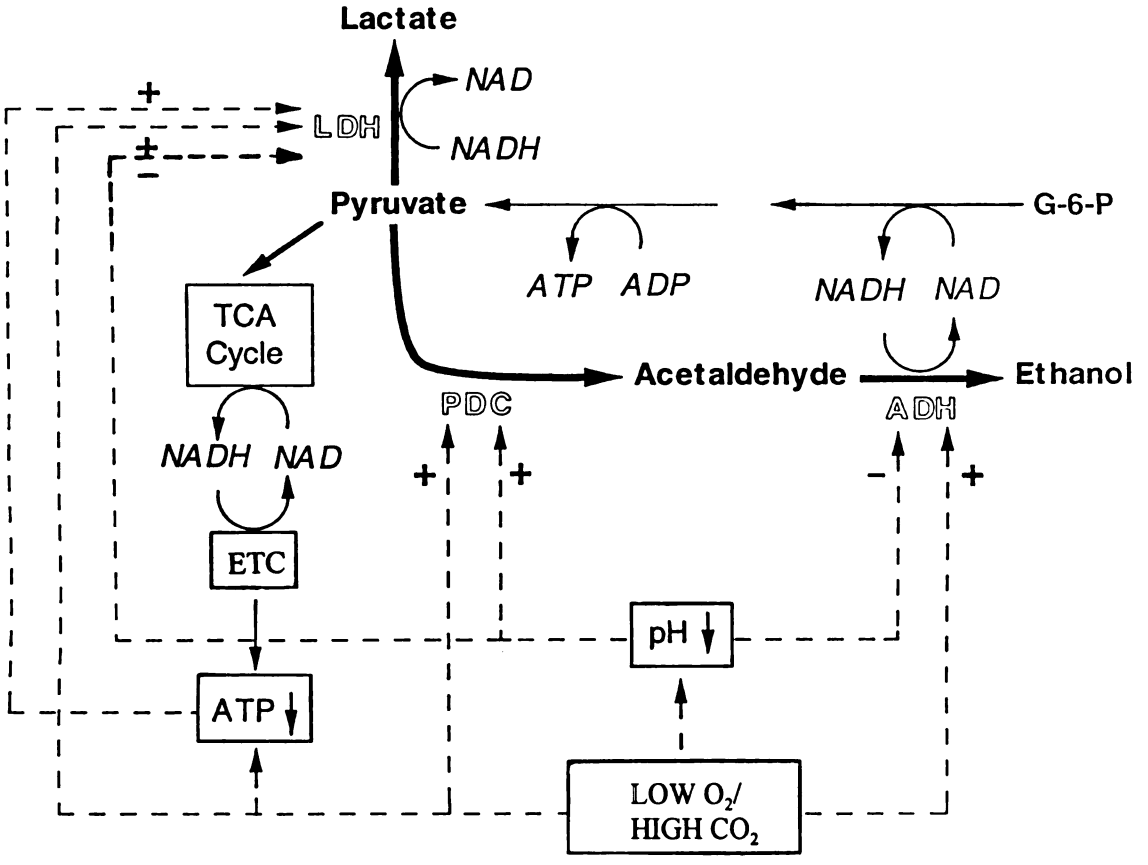

Figure 2.1. Proposed pathway of fermentative metabolism is asparagus spears regulated by low O_2/high CO_2 stress. ADH (alcohol dehydrogenase); ETC (electron transport chain); G-6-P (glucose-6-phosphate); LDH (lactic dehydrogenase); PDC (pyruvate decarboxylase); TCA (tricarboxylic acid cycle); ---+---> , induction and /or activation; ---_--->, reduction and/or inhibition. Adapted from Ke et al. (1995).

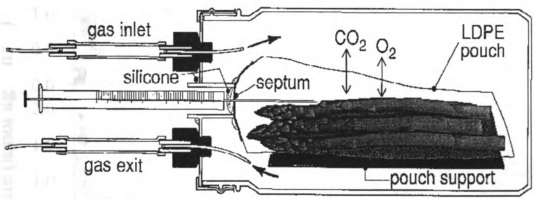

Figure 2.2. Schematic drawing of experimental apparatus allowing adjustment of a package gas composition to target levels by controlling composition of the atmosphere in the exterior chamber.

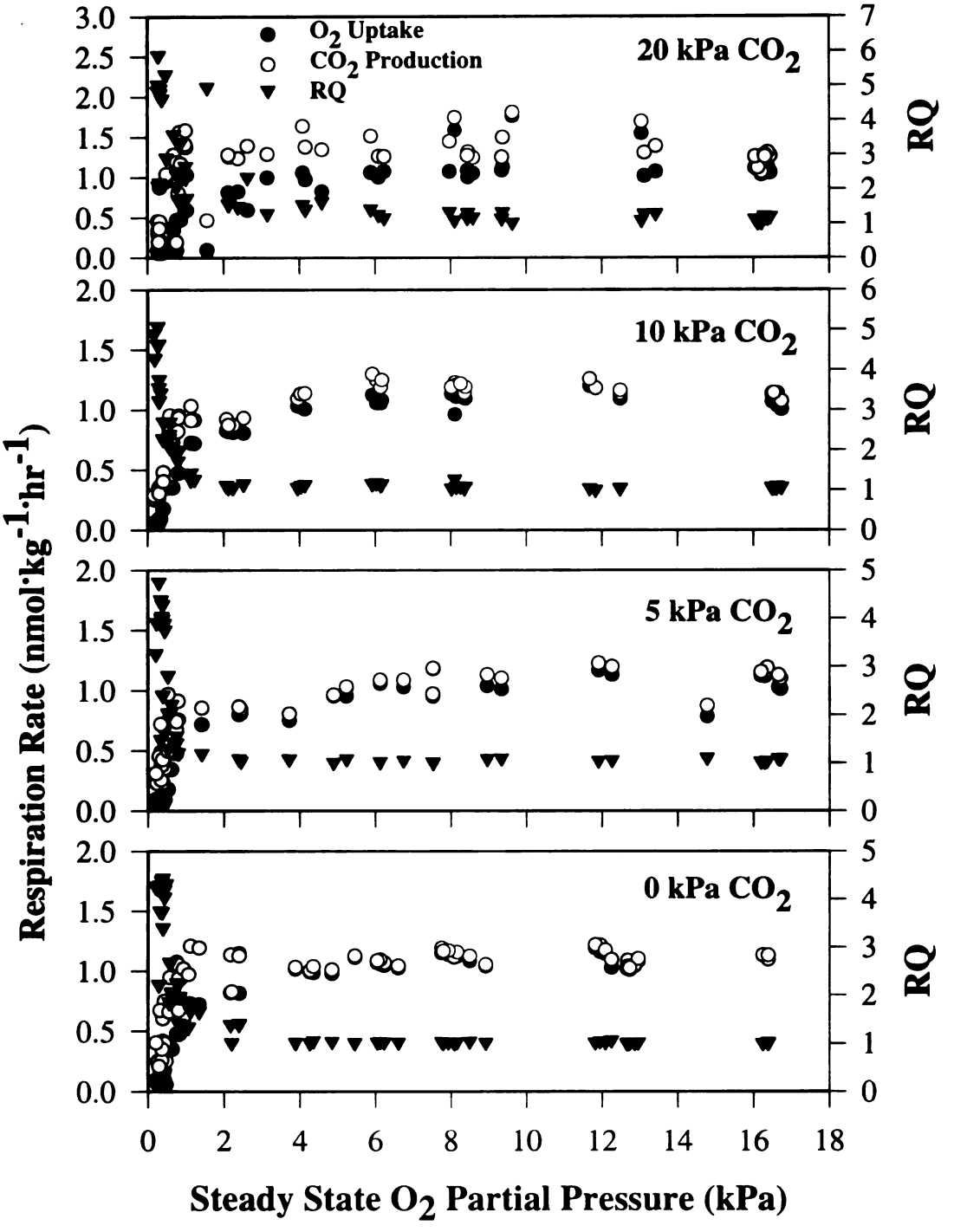

Figure 2.3. The effects of storage O_2 and CO_2 on O_2 uptake, CO_2 production, and respiratory quotient (RQ) in harvested asparagus spears held at 1 °C for 6 days.

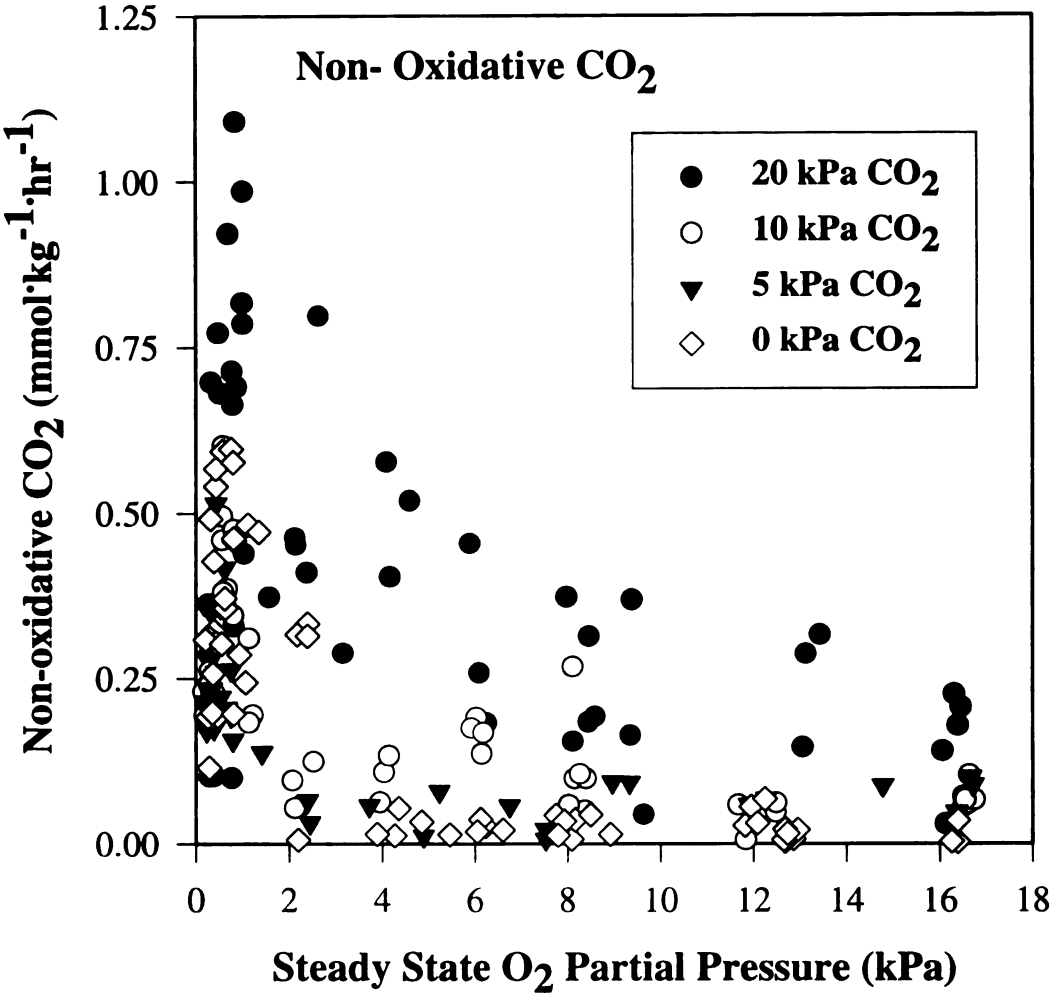

Figure 2.4. The effects of storage O_2 and CO_2 on non-oxidative CO_2 in harvested asparagus spears held at 1 °C for 6 days.

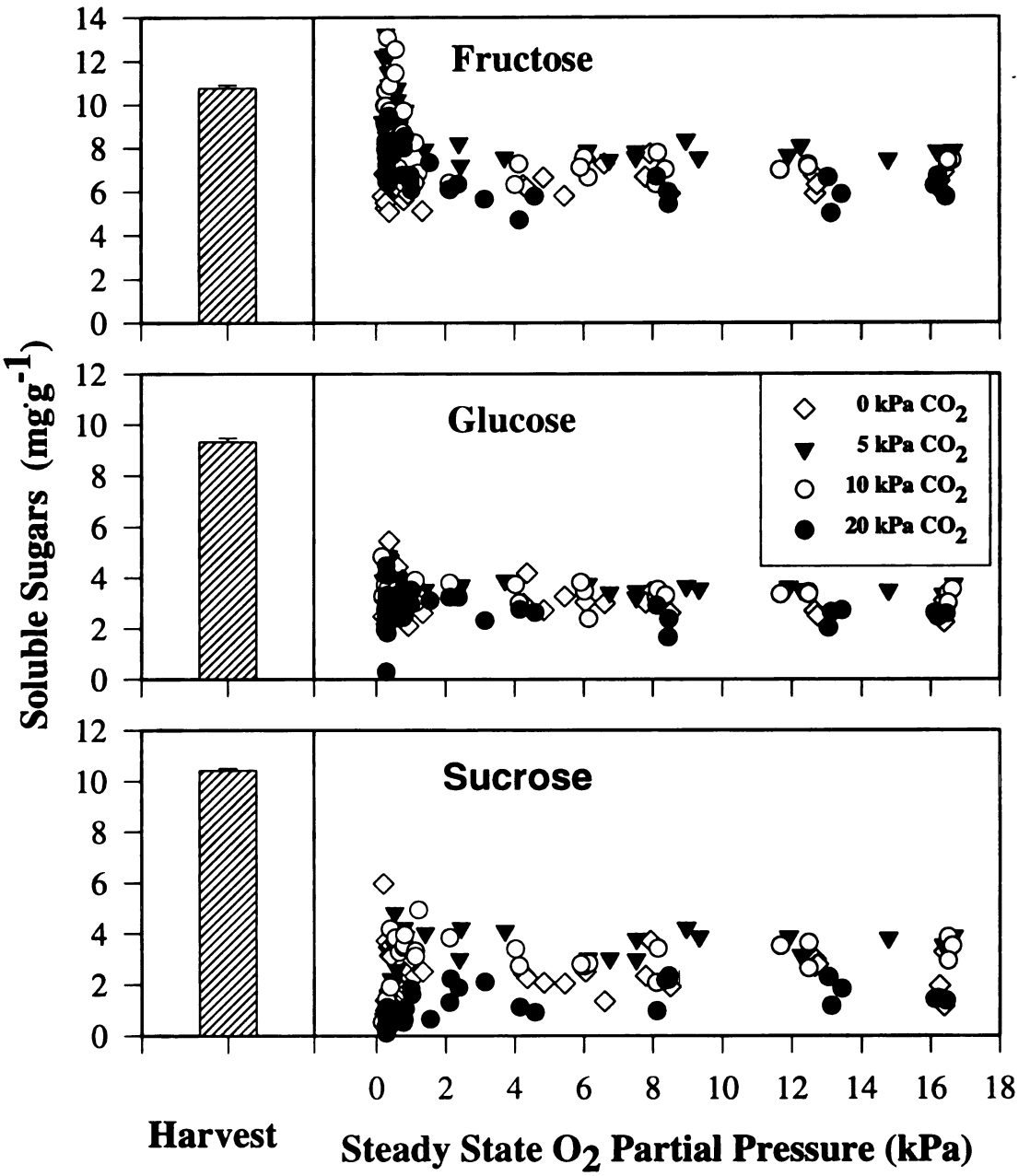

Figure 2.5. The effects of storage O_2 and CO_2 on the contents of fructose, glucose, and sucrose (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

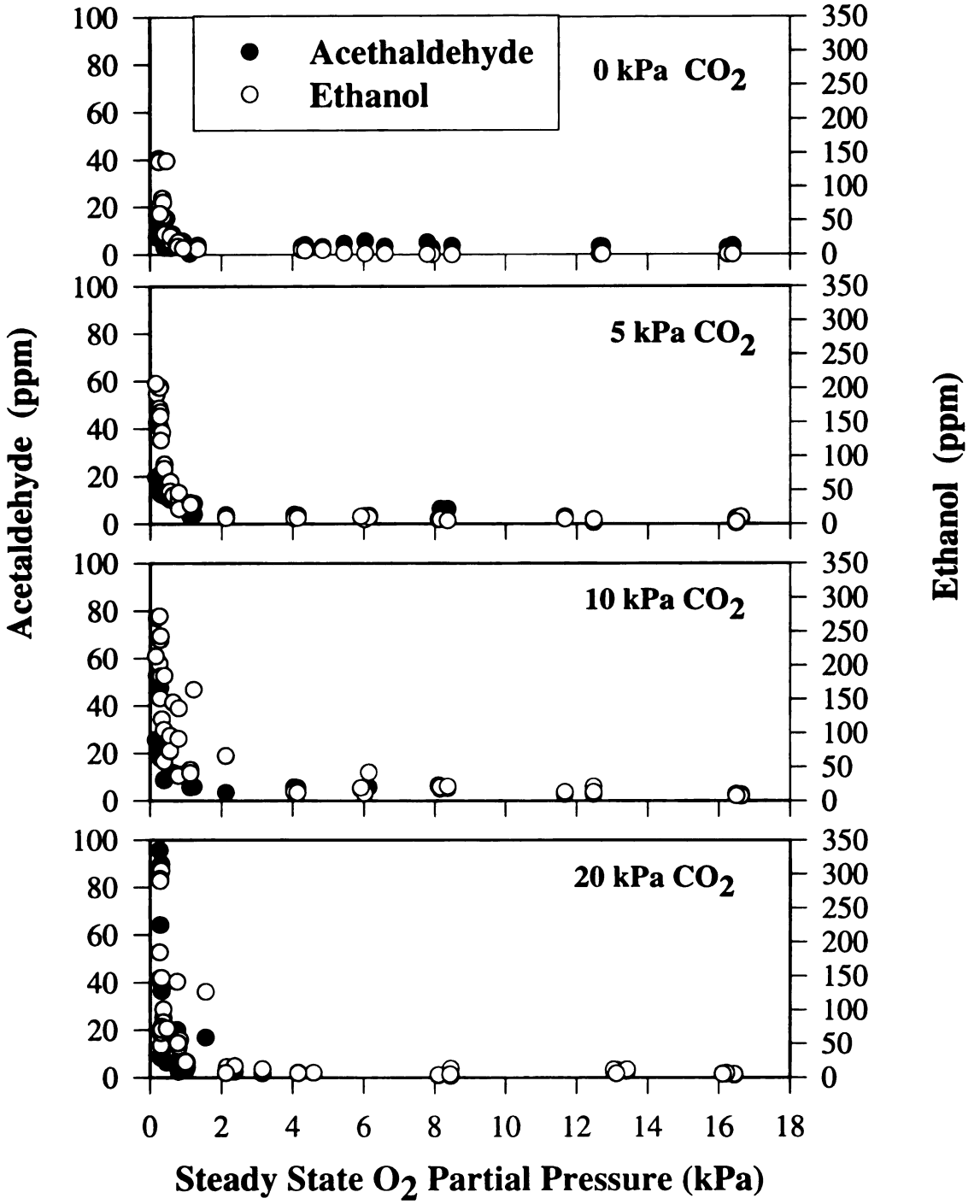

Figure 2.6. The effects of storage O_2 and CO_2 on package headspace acetaldehyde and ethanol for asparagus spears held at 1 °C for 6 days.

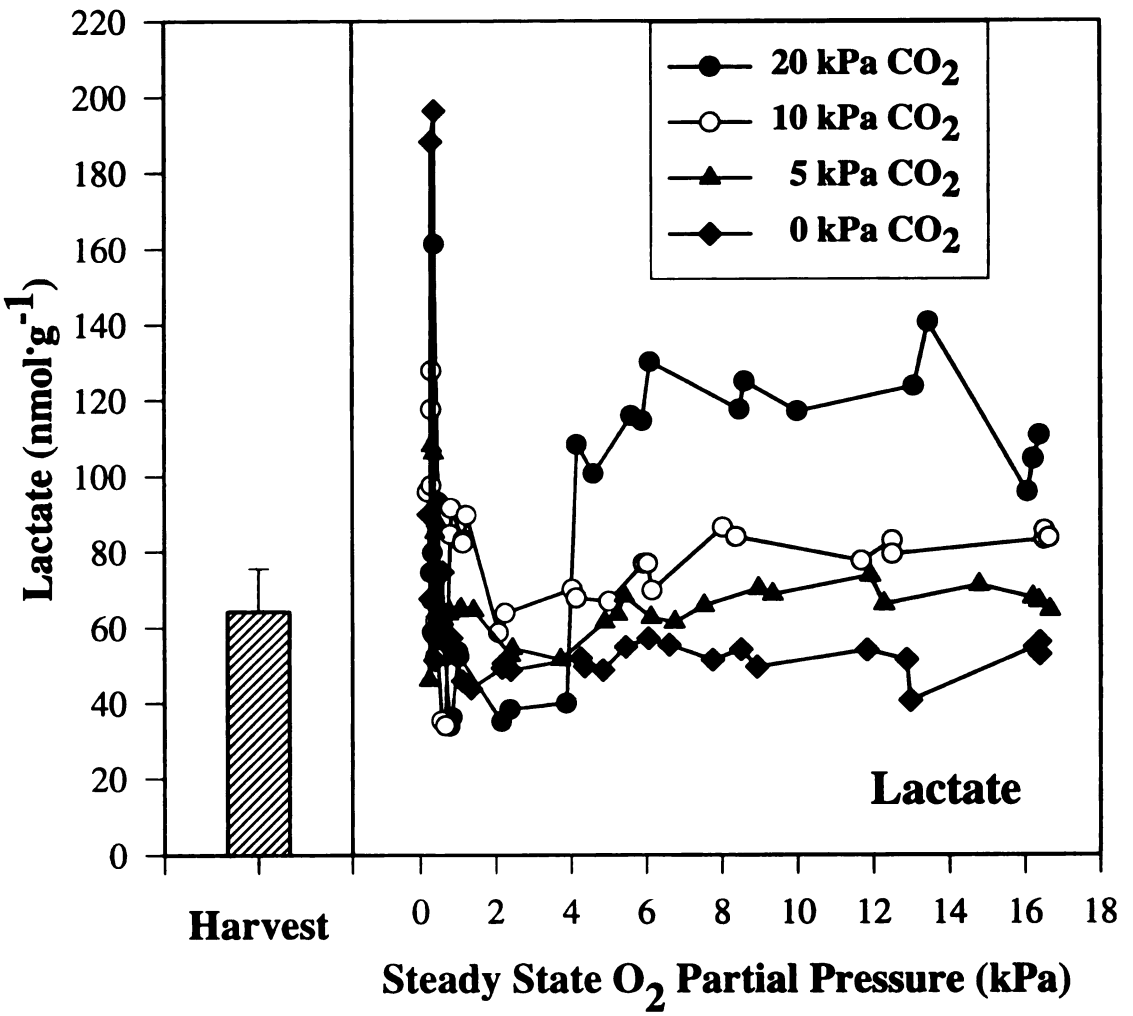

Figure 2.7. The effects of storage O_2 and CO_2 on the content of lactate (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

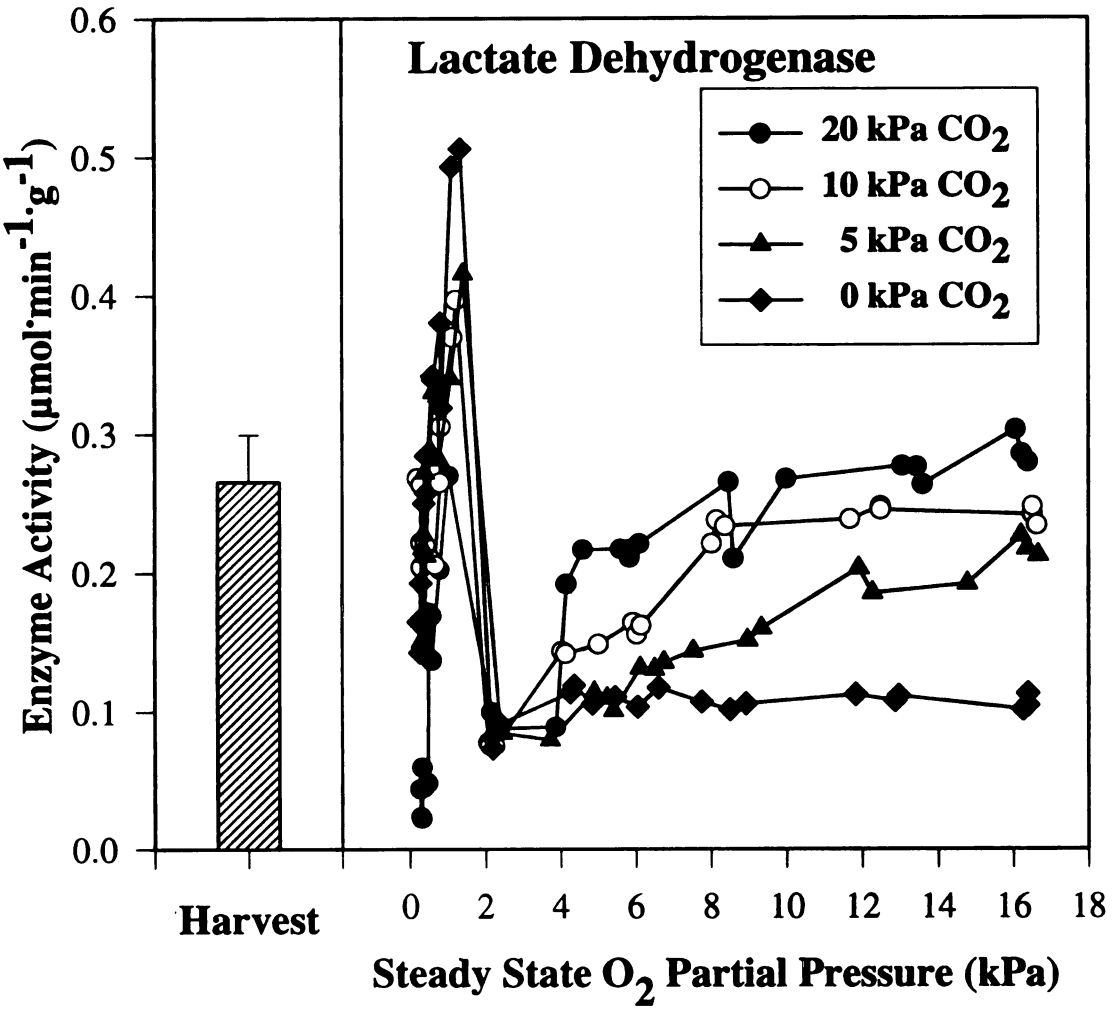

Figure 2.8. The effects of storage O_2 and CO_2 on the activity of lactate dehydrogenase (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

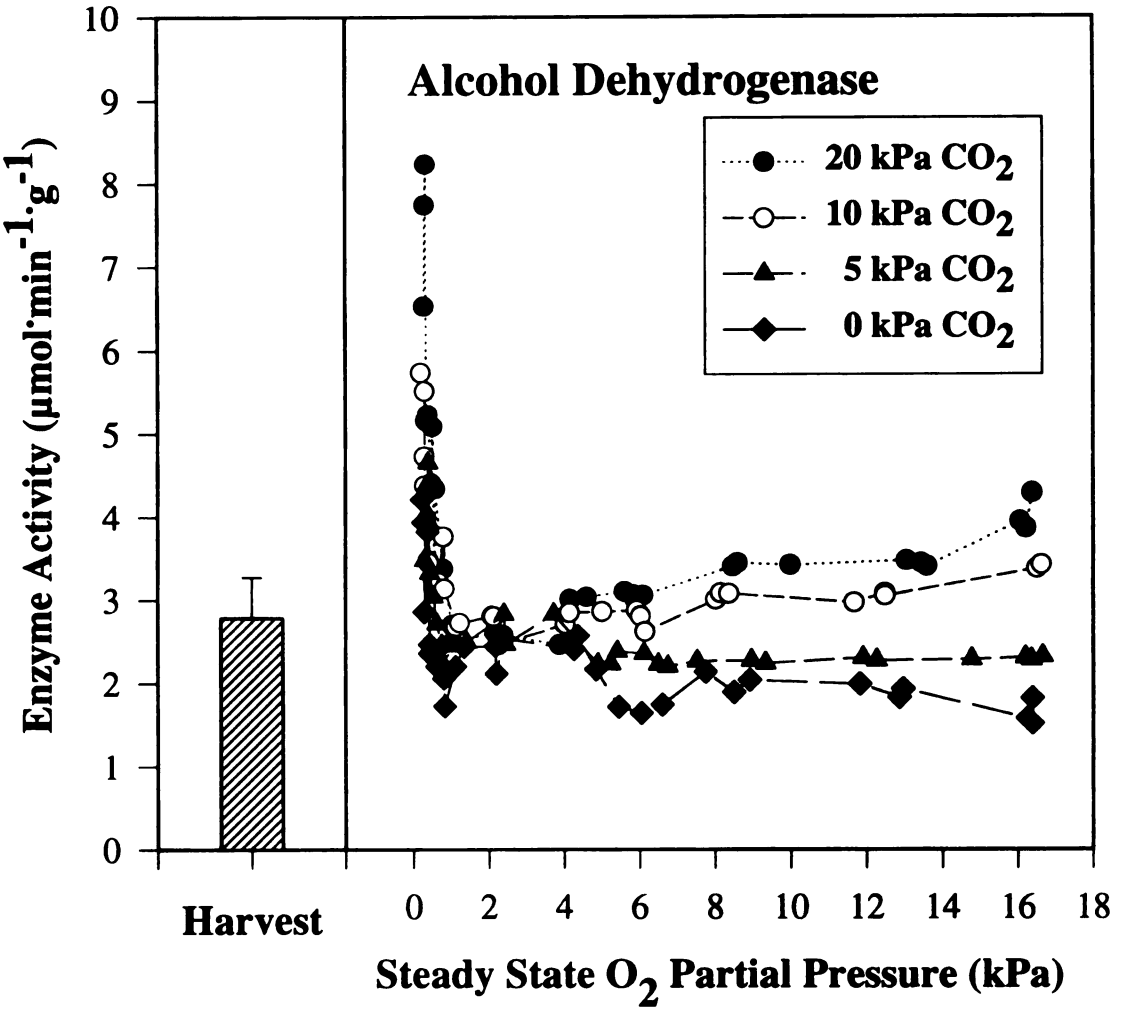

Figure 2.9. The effects of storage O_2 and CO_2 on the activity of alcohol dehydrogenase (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

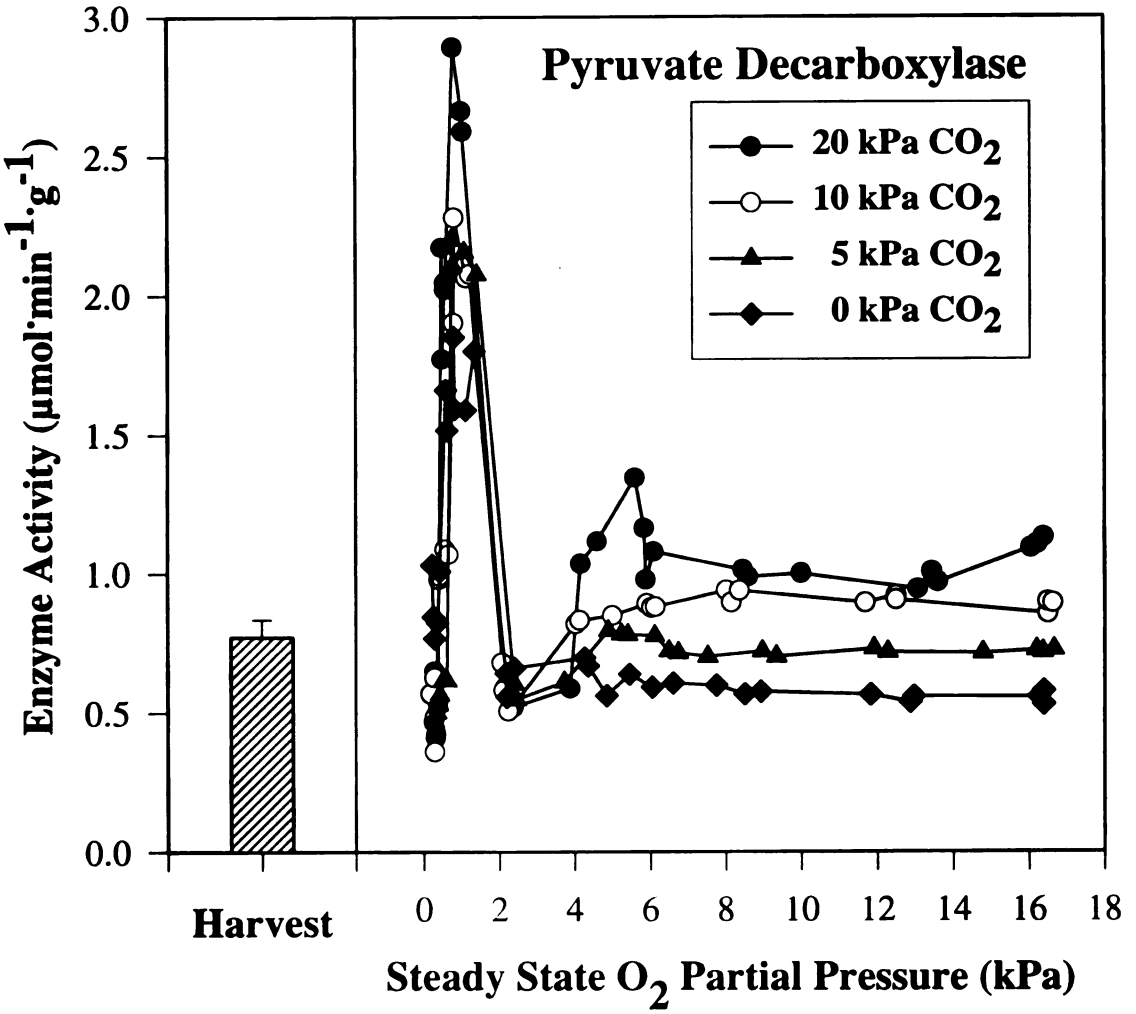

Figure 2.10. The effects of storage O_2 and CO_2 on the activity of pyruvate decarboxylase (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

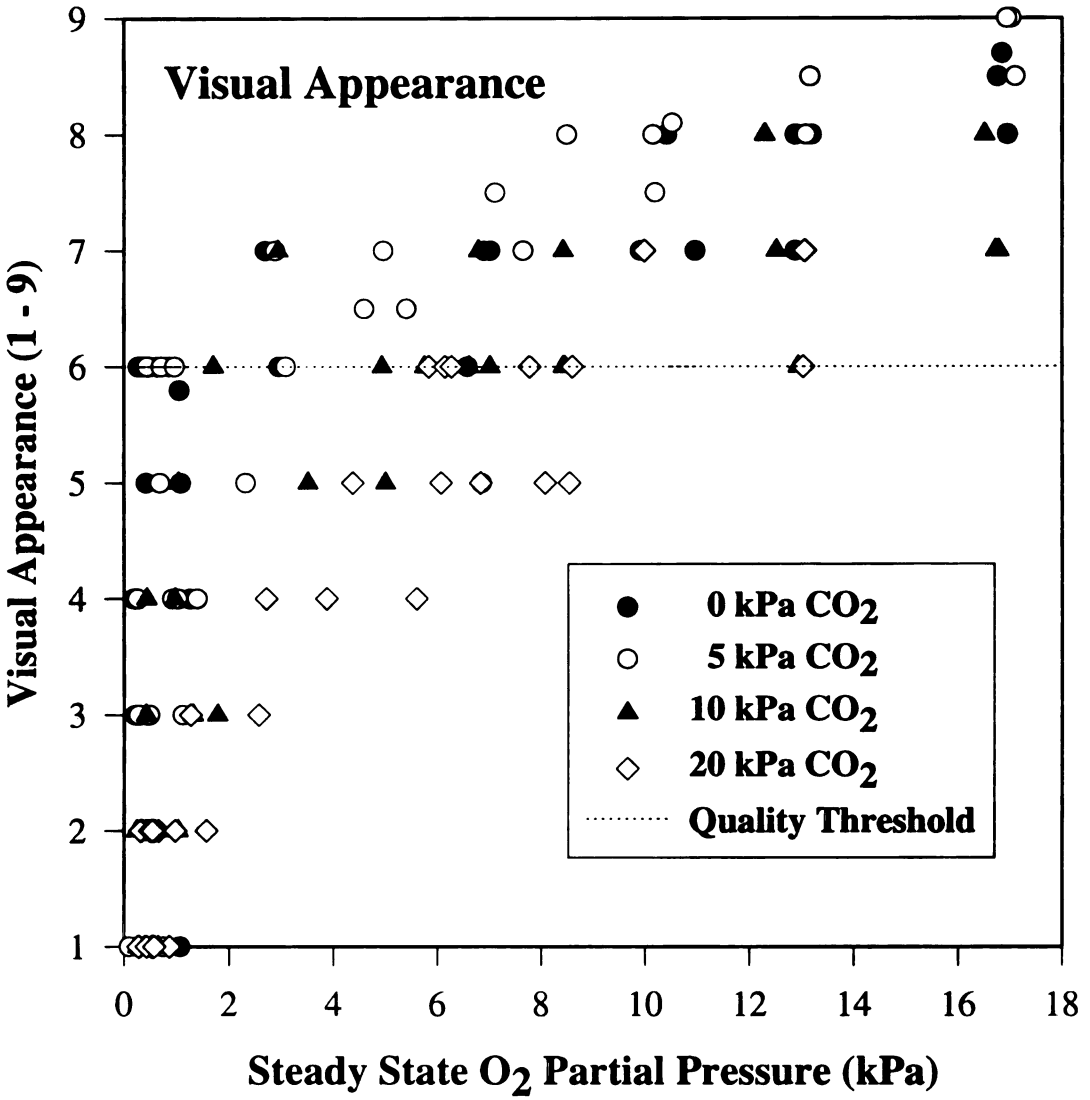

Figure 2.11. The effects of storage O_2 and CO_2 on visual appearance in asparagus spears held at 1 °C for 6 days. Value 6 represents the visual appearance threshold.

CHAPTER 3

REGULATION OF THE STEADY STATE O_2 IN ASPARAGUS SPEARS TIPS: RESPONSES OF GLYCOLYTIC METABOLISM AND SUCROSE-METABOLIZING ENZYMES UNDER LOW O_2/HIGH CO_2 ATMOSPHERES

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) spears are rapidly developing shoots when harvested, with meristematic tips that possesses intense metabolic activity. Harvesting separates the carbohydrate source from the developing shoots with the results that spear tips are nearly depleted of sucrose soon after harvest. This sucrose loss is suspected to limit shelflife and is particularly accentuated in the spear tips (Irving and Hurst, 1993).

Sugars, primarily sucrose, are capable of acting as regulatory signals that affect gene expression, and thus, metabolism in all organisms (Koch et al., 1996). However, as the sugar levels do not always correlate with sugar-regulated gene expression, it is suggested that metabolic flux, rather than accumulated sugar levels, is the important in triggering sugar-responsive gene expression (Jang and Sheen, 1997). Degradation of sucrose within plant cell involves sucrose synthase (SS, EC 2.4.1.13) and/or invertases (IN, EC 3.2.1.26). These enzymes are present in all plant tissues and found at high levels particularly in sink tissues (Avigad, 1982). The invertase reaction produces glucose and fructose, which must be phosphorylated before subsequent glycolytic metabolism. In higher plants, phosphorylation of glucose involves hexokinase (HK, EC 2.7.1.1), in a nonspecific reaction, or glucokinase (Turner et al., 1977a). Phosphorylation of fructose is apparently catalyzed by a specific fructokinase (FK, EC 2.7.1.4) (Turner et al., 1977b).

Carbohydrates are the principal energy source for most living organisms, and the main pathway of carbohydrate degradation is through glycolysis (Plaxton, 1996) (Fig. 1.2., p.20). The glycolytic pathway serves in three fundamental roles: to oxidize hexoses to generate carbon skeletons for building new molecules, to produce energy for cellular maintenance and growth, and to generate hexoses in gluconeogenesis (Plaxton, 1996). Glycolysis shares some steps with oxidative pentose phosphate (OPP) pathway. Glucose-6-phosphate from glycolysis can be diverted to OPP to produce gluconate-6-phosphate and NADPH, step catalyzed by glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). The primary role of OPP is the production of NADPH to be used in biosynthetic reactions (Ap Rees, 1980).

The first rate-limiting step in glycolysis is the interconversion of fructose-6-P (F6P) and fructose-1,6-P_2 (F1,6P_2), a reaction catalized by these enzymes. ATP:phosphofructokinase (ATP:PFK, EC 2.7.1.11) operates in the glycolytic direction (Plaxton, 1996), PFK, PPi:phosphofructokinase (PPi:PFP, EC 2.7.1.90) catalyzes the reversible conversion of F6P to F1,6P_2 (Smith et al., 1984). Another rate- limiting step in glycolysis is the conversion of phosphoenolpyruvate (PEP) to pyruvate (PYR) by pyruvate kinase (PK, EC 2.7.1.40) (Plaxton, 1996). Glycolytic flux may be regulated at either or both of these two steps.

Exposure of harvested horticultural crops to low O_2/high CO_2 environments can reduce deterioration and influence quality and storage life by reducing metabolic activity (Kader, 1986). However, much has yet to be understood regarding on the biochemical effects of the application of combined levels of these two gases. A reduced metabolic rate

may lead to reduced sugar depletion in asparagus during storage. The evaluation of the changes in the metabolite pools and activities of sucrose-metabolizing and glycolytic enzymes in crops exposed to low O_2/high CO_2 levels may provide information of the control of glycolytic carbon flux. Thus, it must help to elucidate the processes through which sugars are utilized and the mode of action of low O_2/high CO_2 in a commodity.

Our objective was to investigate how glycolytic intermediate pools and activity of the sucrose-metabolizing and glycolytic enzymes in these tissues were influenced by low O_2/high CO_2 partial pressures. These data were to be used to evaluate the mechanisms governing carbon flux though glycolysis and to identify reactions which may exert significant control over sugar content in asparagus spear tips stored under the above conditions at 1 °C.

MATERIAL AND METHODS

Plant material. Asparagus spears (*Asparagus officinalis* L. cv. Jersey Giant) were harvested early in May from the Michigan State University Horticultural Research Center between 6:00 and 9:00 a.m. and stored at 1 °C. Samples were taken for analyses at harvest and after storage. Spears selected to represent harvest conditions were frozen in liquid N_2 in the field. Spears were harvested when they reached a minimum length of 240 mm and were trimmed to 180 mm. Only straight undamaged spears with closed bracts and no obvious symptoms of disease were used. Spears to be placed on storage were chilled with ice while still in the field and transported to the laboratory within the hour. After storage, spears were frozen in liquid N_2. After freezing, all spears were stored at -80 °C until analysis. Three replicate experiments were conducted in consecutive asparagus

seasons (1995, 1996, 1997). Samples comprised the most apical 45 mm of the spear.

Atmosphere generation. O_2 partial pressures ranging from a low of a 0.18 kPa to a high of 16 kPa were generated in packages for four CO_2 treatment levels (0, 5, 10, and 20 kPa). O_2 levels were obtained by sealing spears in packages composed of low density polyethylene (LDPE) of varying film thickness and placing the packages in air (0 kPa CO_2) or enclosing the packages in glass chambers with 20.7 kPa O_2 and 5, 10, and 20 kPa CO_2 (Beaudry, 1993). Chamber lids were fitted with inlet and outlet ports for purging with gas mixtures; a glass tube (1.3 cm i.d. x 2.7 cm long) was inserted through the lid and glued to a piece of electrical tape on the surface of the enclosed package with silicone sealant (Beaudry, 1993). The silicone sealant formed a septum through which gas samples could be obtained from within the package. Chambers were continually purged with gas mixture of O_2, CO_2, and N_2 gases at 30 $mL \cdot min^{-1}$ to generate the target atmospheres within the enclosed packages. Actual CO_2 levels in the packages were slightly higher than reported values, but for clarity of presentation, are reported as treatment values. The gas composition of individual packages was monitored until steady state conditions were reached. Steady state conditions were achieved on day six of the study. O_2 and CO_2 gradients across the film were used to calculate the flux for both gases through the film using previously obtained permeability data (Lakakul, 1994). Flux data were used to calculate the rates of O_2 uptake and CO_2 production (Cameron et al., 1994).

Glycolytic intermediate extraction. Approximately 5 g of frozen tissue per sample were transferred to liquid N_2 in a precooled mortar and ground with a pestle to a fine powder. Ten mL of ice-cold 0.8 N (v/v) $HClO_4$ were added to the powder, which was

extracted for 30 min at 0 °C with occasional shaking, followed by centrifugation at 30,000 *g* for 30 min at 0 °C. Supernatant pH was adjusted to pH 7.0 to 7.2 with 5 N K_2CO_3, allowed to sit on ice for 10 min, and clarified by centrifugation at 30,000 *g* for 10 min. The resulting supernatant was used for analysis of intermediates. To estimate losses of metabolites during extraction, measured amounts of pure glycolytic intermediates roughly equal to the amounts present in the tissue, were added to the extracting medium. Recoveries were 96%, 95%, 90%, 89%, 87%, 89%, 90%, 96%, 92% for G6P, F6P, F1,6P, DHAP, 3PGA, 1,3PGA, 2PGA, PEP, and PYR, respectively. The recovery assays were replicated in six times.

Glycolytic intermediates assay. The glycolytic intermediates were assayed spectrophotometrically (Hitachi, Model U3000, Hitachi Inc., Tokio, Japan) at 25 °C using a temperature controlled circulator (Polystat, Cole-Palmer Instrument Co., Chicago, IL). The oxidation/reduction of pyridine nucleotides was assayed by measuring changes in absorbance of the assay cocktails at 340nm according to procedures adpted from Kato-Noguchi and Watada (1986), Kerbel et al. (1988), and Bergmeyer (1983). Measurements were taken for 15 to 20 min in 1mL of triethanolamine buffer (TEA, pH 7.4) and other additions specified for each intermediate. Depending on the intermediate, 50 to 100 µL enzyme extract was used. Assay cocktails for the intermediate were as follows*: Glucose-6-Phosphate* (G6P): 250 mM TEA, 0.25 mM of NADP, 4 mM $MgCl_2$, 0.5 units glucose-6-P dehydrogenase; *Fructose-6-phosphate*(F6P): the above mixture for G6P plus 1 unit of phosphoglucose isomerase; *Glyceraldehyde-3-Phosphate* (GAP): 250 mM TEA, 20 mM EDTA, 0.13 mM NADH, 0.5 units glycerol-3-phosphate dehydrogenase (GDH);

Dihydroxyacetone phosphate (DHAP): the previous mixture for GAP plus 1.5 units of triosephosphate isomerase (TIM); *Fructose-1,6-bisphosphate* ($F16P_2$): the previous mixture for DHAP plus 1 unit of aldolase; *Glycerate-1,3-bisphosphate* (1,3PGA): 200 mM TEA, 1mM EDTA, 0.13 mM NADH, and 10 units glyceraldehyde-3-phosphate dehydrogenase (GAPDH); *Glycerate-3-phosphate* (3PGA): 200 mM TEA, 2 mM EDTA, 0.13mM NADH, 8 mM Na_2 SO_4, 8 mM ATP, 4 units of GAPDH, 1 unit GDH, 25 units of TIM, 16 units phosphoglycerate kinase; *Pyruvate* (PYR): 200 mM TEA, 1mM EDTA, 0.13 mM NADH, 12 mM Mg SO_4, 45 mM KCl, 2 mM ADP, and 10 units lactate dehydrogenase; *Phosphoenolpyruvate* (PEP): the previous mixture for PYR plus 5 units of pyruvate kinase; *2-Phosphoglyceric acid* (2PGA): the previous mixture for PEP plus 4 units of enolase.

Sucrose synthase and invertase extraction solutions. The solutions used for sucrose synthase (SS, EC 2.4.1.13) and invertase (EC 3.2.1.26) extractions were modifications from Irving and Hurst (1993). Solution A contained 40 mM Hepes-NaOH (pH 7.6), 2 mM Na_2EDTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 10% of glycerol. Solution B was buffer A minus PMSF and Solution C was Solution B containing 1 M NaCl.

Sucrose synthase and soluble invertase extractions. Approximately 5 g of frozen tissue, to which 50 mg/g tissue of insoluble (40,000 mw) PVPP was added, was ground as described previously and mixed with 10 mL of ice-cold Solution A. The mixture was centrifuged at 30,000 *g* for 30 mim. The supernatant was collected and 1.3-mL samples were passed through a column of of Sephadex G-25 (Pharmacia Uppsala, Sweden)

medium-sized beads (9 mL bed volume), previously swollen overnight in 40 mM Hepes-NaOH (pH 7.6) and equilibrated in Solution B (Helmerhorst and Stokes, 1980; Neal and Florini, 1973) for removing salt and sugars. The eluate was assayed for sucrose synthase and acid and neutral soluble invertase activities. Substrates and coupling enzymes were purchased from a commercial source (Sigma Chemical Co., St. Louis, MO).

Bound acid invertase extraction. Two-hundred milligrams (fresh mass basis) of the cell debris collected from the bottom of the centrifuge tube described previously were transferred to 2 mL microcentrifuge tubes and resuspended (30 sec vortex) in 1 mL of Solution A. After microcentrifuging at 3,000 *g* for 30 sec, the pellet was washed three times with 1 mL of Solution B. The pellet was resuspended (1 min vortex) in 1mL of Solution C and kept overnight at 4 °C to release bound protein. The supernatant was assayed for acid invertase activity.

Sucrose synthase and invertase assays. Assays for enzyme activities were assayed in a final volume of 1 mL based on procedures adapted from Huber and Akazawa (1986). Blanks were prepared for each assay. The change in absorbance was monitored spectrophotometrically (Hitachi, Model U3000, Hitachi Inc., Tokio, Japan, double-bean) at 30 °C by following the oxidation/reduction of pyridine nucleotides at 340nm. Continuously monitored reactions were linear for at least 5 min under the assay conditions. Activities of all enzymes were expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$ of tissue on fresh mass basis.

The sucrose synthase cocktail included: 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$, 1 mM EDTA, 25 mM sucrose, 0.13 mM of NAD, 3 mM UDP (omitted from the

blank), 1mM ATP, 15 mM KCl, 25 units phosphoglucose isomerase, 10 units glucose-6-phosphate dehydrogenase, and 5 units hexokinase. The reaction was started by addition of 75 µL extract.

Acid invertase: 50 mM sodium acetate (pH 5.0), 2 mM $MgCl_2$, 0.13 mM NAD, 20 units phosphoglucose isomerase, 5 units glucose-6-P dehydrogenase, 5 units hexokinase, 1 mM Mg.ATP, 100 µL of extract, and 33 mM sucrose (started the reaction). The reaction mixture was incubated for 30 min at 30 °C. The reaction, which was linear with respect to time and volume of extract, was stopped by boiling for 5 min. Blanks contained all the above and boiled enzyme.

Neutral invertase (NI, EC 3.2.1.26): The reaction was the same as for acid invertase except that the buffer was 50 mM Hepes-NaOH (pH 7.0).

Glycolytic enzymes extraction. Approximately 5 g of frozen tissue per replicate was ground as described above and mixed with a 10 mL ice-cold solution containing 60 mM Tris-Hcl (pH 7.6), 5 mM Na_2EDTA, 10 mM $MgCl_2$, 5 mM of dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 10 % (v/v) glycerol, and 50 mg/g tissue of insoluble (40,000 mw) PVPP. The mixture was centrifuged at 30,000x *g* and part of the supernatant used immediately for the measurement of the activities of glycolytic enzymes.

Glycolytic enzyme assays. The change in absorbance was monitored spectrophotometrically 30 °C by following the oxidation/reduction of pyridine nucleotides at 340nm. The reaction mixture (1mL) for all assays contained 60 mM Hepes-NaOH (pH 7.4), 2 mM $MgCl_2$, 1 mm EDTA, 15 mM KCl, and other additions specified for each enzyme. Depending on the enzyme, 50 to 75 µL of extract per reaction was used. The

enzyme assay cocktails are as follows: hexokinase (HK, EC 2.7.1.1): 0.13 mM NAD, 4 mM ATP, 15 units glucose-6-phosphate dehydrogenase, and 15 mM glucose (omitted from the blank, otherwise used to start the reaction). Fructokinase (FK, EC2.7.1.4): 0.13 mM NAD, 7 mM UTP, 10 units glucose-6-phosphate dehydrogenase, 15 units phosphoglucose isomerase, and 15 mM fructose (omitted from the blank, otherwise used to start the reaction). ATP-dependent phosphofructokinase: 2 mM $MgCl_2$, 0.2 mM NADH, 1mM ATP, 5 units aldolase, 6 units glycerol-3-phosphate dehydrogenase, 20 units triosephosphate isomerase, and 3 mM fructose-6-phosphate (omitted from the blank, otherwise used to start the reaction). PPi-dependent phosphofructokinase: 2 mM $MgCl_2$, 0.2 mM NADH, 1 mM inorganic pyrophosphate (PPi used to start the reaction), 5 units aldolase, 6 units glycerol-3-phosphate dehydrogenase, 20 units triosephosphate isomerase, 3 mM fructose-6-phosphate, and 20 μM fructose-2,6-bisphosphate (omitted from the blank). Pyruvate kinase: 0.2 NADH, 1.5 mM ADP (omitted from the blank), 15 mM KCl, 10 units lactate dehydrogenase, and 1,5 mM phosphoenolpyruvate (to start the reaction). Glucose-6-phosphate dehydrogenase: 0.25 mM NADP and 2 mM glucose-6-phosphate (omitted from the blank). The reaction was started by addition of extract.

The recovery of enzyme activity through the quantification process was above 91% and below 99%, as determined by 3 to 5 repeated assays with pure enzymes added to the extraction medium.

Crossover plot. A crossover plot (Chance et al., 1958; Kerbel et al 1988) was used to evaluate stimulation or inhibition of the irreversible reactions regulating the rate of respiration. Crossover usually ranges from -1 to +1. Data on the 0 line indicates that

there is no difference between treatments. In this research, the broad range of O_2 partial pressures combined with four CO_2 levels allowed, by a crossover evaluation of the intermediate pools, separations of the effects of O_2 and CO_2 to detect the control points of glycolysis in asparagus spears tips. Two crossover plot were constructed: 1. comparing the highest O_2 level (16 kPa O_2), with O_2 levels below that for each of the four CO_2 treatments (Fig. 3.7); and 2. comparing 0 kPa CO_2 with higher CO_2 treatments (Fig. 3.8).

RESULTS

Sucrose-metabolizing enzymes. SS and BAI activities were higher than those of AI and NI at harvest (Fig. 3.1). From 2 to 16 kPa O_2, at 20 kPa CO_2, however, BAI activity was maintained higher than SS. After 6 d at 1 °C, the spears stored at near ambient atmosphere (16 kPa O_2, 0 kPa CO_2) experienced an increase in the invertase, but no change in SS activity. However the activities AI, BAI, NI, and SS were markedly reduced at the lowest O_2 levels, for all CO_2 treatments (Fig. 3.1). In addition, the activities of these sucrose-metabolizing enzymes were altered by CO_2 treatments. AI, BAI, and SS activities were enhanced by 20 kPa CO_2 relative to 0 kPa O_2. The activities of these enzymes was reduced by 5 kPa CO_2, which is consistent with higher sucrose content found under similar conditions (Silva et al., 1998a). Interestingly, NI was not affected in the same manner by CO_2; its activity was reduced by 5 and 20 kPa CO_2 and enhanced by 10 kPa CO_2.

Intermediates. Of the hexose-phosphates, G6P was present in the highest level at harvest and accumulated to the greatest extent (≈ 3-fold) for spears stored at near ambient

atmosphere (0 kPa CO_2, 16 kPa O_2) (Fig. 3.2). O_2 partial pressure below 1 kPa caused a decline in the contents of G6P, F6P, and $F16P_2$, for all CO_2 treatments. G6P and F6P contents decreased as CO_2 levels increased.

$F16P_2$ was not affected by storage at 1 °C. O_2 partial pressures below 1 kPa caused a reduction in $F16P_2$ for all CO_2 treatments. $F16P_2$ content was enhanced by 10 and 5 kPa CO_2 but reduced by 20 kPa CO_2. The ratio G6P/F6P was approximately 4 at harvest, which is near equilibrium (Fig. 3.3). This ratio declined with storage and with increasing CO_2 partial pressures. At O_2 levels below 1 kPa, however, G6P/F6P ratio tended to increase as CO_2 increased.

The $F16P_2$/F6P ratio declined relative to harvest (Fig. 3.3). This ratio increased slightly as CO_2 partial pressures increased. At O_2 levels below 1 kPa, the $F16P_2$/F6P ratio increased, declining again below 0.5 kPa O_2.

The GAP and DHAP contents after storage were similar to those at harvest (Fig. 3.4). The content of these triose-P intermediates declined as the O_2 partial pressure dropped below 4 kPa. The amount of GAP and DHAP was not affected by CO_2.

The contents of 1,3-PGA, 3-PGA, and 2-PGA tended to increase with storage at near ambient O_2 and CO_2 levels. Their content tended to decline as CO_2 partial pressure increased. At O_2 partial pressures greater than 1 kPa, the content of these intermediates was largely unaffected by O_2, with the possible exception of 3-PGA for spears treated with 20 kPa CO_2. Below 1 kPa O_2, the contents of 1,3-PGA, 3-PGA, and 2-PGA were dramatically (Fig. 3.5).

The contents of PYR was approximately 1/3 that of PEP at harvest (Fig. 3.6).

Following storage, this ratio declined to 1 for spears stored at near ambient atmospheres. The content of both compounds declined approximately 50% as CO_2 increased to 20 kPa. The effect of O_2 on both intermediates was complex. PEP tended to be enhanced by O_2 levels between 0.5 and 2 kPa. The PYR/PEP tended to decline between 2 and 4 kPa O_2 relative to higher O_2 levels; this ratio tended to increase between 1 and 2 kPa O_2.

A crossover plot shows that, for all of CO_2 treatments, the interconversion of PEP and PYR was the only step in glycolysis to result in a crossover event (Fig. 3.6). A crossover took place only at O_2 levels below 1.5 kPa. As the O_2 level declined, the levels of intermediates declined and the effect tended to be greater for those intermediates 'earlier' in the glycolytic pathway. When comparing higher CO_2 (20, 10, and 5 kPa) to the lowest (0 kPa) CO_2 level no clear pattern was observed (Fig. 3.8 A,B, and C). The most obvious crossover event was detected at the conversion of F6P and $F1,6P_2$, at higher O_2 levels for 5 and 10 kPa CO_2. $F1,6P_2$ tended to accumulate relative to F6P under these atmospheric conditions.

Glycolytic enzymes. HK activity increased 4-fold with storage under near-ambient conditions (Fig. 3.9). Relative to harvest, the activity was markedly higher for 0 kPa CO_2 followed in order by 10, 5, and 20 kPa CO_2. Activity declined as O_2 below 4 kPa.

Compared to HK, FK activity was about 10 times higher at harvest (Fig. 3.9). However, the activity of FK declined with storage. The effects of CO_2 and O_2 were nearly identical for FK and HK, with activity of FK declining in the same order in response to CO_2 and declining as O_2 dropped below 4 kPa.

G6PDH activity increased slightly relative to harvest for spears stored under near

ambient conditions (Fig. 3.9). Activity declined as CO_2 increased in the O_2 range of 4 to 16 kPa. The effect of O_2 was complex. As O_2 declined below 8 kPa to 2 kPa O_2, G6PDH activity declined. Below 2 kPa O_2, there was an increase in activity, which peaked from 0.5 to 1 kPa O_2 and declined as O_2 declined further.

ATP:PFK activity declined by approximately 1/3 compared to harvest for spears stored under near-ambient atmospheric conditions (Fig. 3.10). ATP:PFK activity declined as CO_2 partial pressures increased. ATP:PFK activity also declined as O_2 partial pressure decreased. The O_2-dependet decline was greatest below 2 kPa O_2 for all CO_2 treatments except the 20 kPa treatment, which exhibited marked inhibition below 6 kPa O_2.

At harvest, the activity of PPi-PFK was approximately 4-fold higher than ATP-PFK (Fig. 3.10). Storage did not appear to affect activity for spears held in near-ambient conditions. The higher activity for PPi:PFK over ATP:PFK was maintained accros the O_2 range in spear tips for all CO_2 treatments. After storage, the highest PPi:PFK was for the 10 kPa CO_2, declining, in order, for 0, 5, and 20 kPa CO_2. The pattern relative to O_2 levels was nearly identical to that for G6PDH activity.

PK activity did not change in response to storage when spears were held in near-ambient atmospheres. Activity declined as CO_2 partial pressures increased (Fig. 3.10). There was a marked inhibition by even 5 kPa CO_2 for spears held in 4 to 14 kPa O_2. For each of the 3 CO_2 treatments, PK activity declined as O_2 declined in the range of 16 to 2 kPa O_2. At 20 kPa CO_2 PK activity was reduced by 1/3. For 0 kPa CO_2 treatment, PK activity declined only below 6 kPa O_2. PK activity increased at O_2 levels below 2 kPa, similarly to G6PDH and PPi:PFK. However, for PK this increase seemed to be most

pronounced for the 20 kPa CO_2 treatment and the increase relative to activity at 2 kPa O_2 was higher when compared to the other two enzymes.

DISCUSSION

Sugar-metabolizing enzymes. In asparagus spears tips, as CO_2 levels increased and O_2 levels decreased the content of sucrose decreased (Silva et al., 1998a). This sugar was most markedly affected during storage. Sucrose is metabolized by sucrose synthase, acid and/or neutral invertases. The genes for these enzymes are modulated by sugars (Koch et al., 1996), and their isozyme forms have contrasting carbohydrate responses (Geiger et al., 1996). In asparagus spear tips, the activity of these enzymes seemed to be affected differently by the availability of sugars and by O_2 and CO_2 levels. In maize, expression of invertase and sucrose synthase genes were not only modulated by the availability of carbohydrates, but also by different genes. For example, expression of the *Ivr1* invertase and *Sh1* sucrose synthase were starvation-tolerant and could be repressed by sugar. In contrast, *Irv2* ans *Sus1* expression was enhanced by increasing sugar availability and markedly sensitive to carbohydrate depletion (Geiger et al., 1996).

The results indicate that in asparagus spear tips, hydrolysis of sucrose to feed glycolytic carbon flux probably occurs mostly in the cytosol (via SS and NI), and in the vacuole and apoplasts (via BAI). In maize seedlings, sucrose synthase is the major sucrose-metabolizing enzyme, which seems to be induced at transcriptional level (Guglielminetti, et al., 1997). In maize leaf, phosphorylation of serine-15 of SS *in vivo* selectively activates the cleavage reaction by increasing the apparent affinity of the enzyme for sucrose and UDP, suggesting that phoshorylation may have regulatory significance

(Huber et al., 1996). The inhibition in activity of NI by higher CO_2 levels, in contrasting with enhanced activity detected for SS and other invertases, suggests an inhibitory CO_2 effect or a reduction of the amount of the enzyme. *In vivo*, changes in the environment can change the pH in the cytoplasm. It is known that pH regulates the activity of key enzymes and metabolic steps. Anaerobiosis induces cytoplasmic pH decrease (Roberts et al., 1984). In addition, CO_2 at concentrations around 1% and above is known to acidify the cytoplasm (Martin et al., 1993).

In sycamore cells, SS synthase has much more affinity for sucrose as compared with NI. Consequently, the SS pathway may be more important when sucrose availability is limiting, as probably in case of asparagus. Sucrose synthase pathway is also more energetically efficient, since it requires the input of three ATP to metabolize one molecule of sucrose to triose-P, compared to four in the invertase pathway (Huber and Akazawa, 1986). Nevertheless, BAI activity was the highest for 0 kPa O_2, and was enhanced relative to harvest, SS was the enzyme with the highest activity at harvest, which is consistent with the metabolic activity in growing asparagus. The data from this experiment strongly suggest that BAI, rather than other invertases, plays the major role in metabolizing the sucrose delivered to the spear tips under storage conditions. The increased BAI activity with storage indicates that a significant part of the sucrose metabolism in asparagus occurs via the apoplast. In tomato fruit, the hydrolysis of sucrose by acid invertase is proposed to influence the rate and extent of sugar storage in fruit by creating a sucrose concentration gradient between the site of phloem unloading and storage cells (Walker et al., 1978). Since sucrose is the one of the major form of

translocated carbon in plants (Ap Rees, 1980), and the compound that initiates sucrolysis and glycolysis in plant cells (Avigard, 1980; Sung et al., 1989), one biochemical determinant of sink strength is the ability of the sink to metabolize sucrose.

Asparagus spear tips are known to be a very active metabolic sink (Irving and Hurst, 1993). When the spear is harvested, sucrose translocation from the crown is suppressed, having the spear to relay on its stored carbohydrate supply to support the tip's high metabolic and biosynthetic demands. According to Sung et al. (1989), tissues where biosynthesis is predominant, the SS activity tends to be greater than invertase activity. In rapidly growing tissues in which the sucrose content was low or rapidly declining, such as in carrots roots, AI activity was higher when compared to NI (Ricardo and Ap Rees, 1970). In case of asparagus tips, the higher activity at harvest detected for BAI and SS relative to AI and NI may be explained as a means to support spear's high metabolic rate and biosynthetic activity.

Intermediates. The sharply reduced levels of G6P, F6P, and $F16P_2$ below 1 kPa O_2 observed in asparagus may indicate an enhancement of glycolytic carbon flux, as also suggested by the increased G6P/F6P and decreased $F16P_2$/F6P ratios relative to harvest at that O_2 range. Typically the ratio of G6P/F6P is 4:1 in the healthy tissue and is near equilibrium for phosphoglucose isomerase (PGI, EC 5.3.1.9), the enzyme catalyzing this reaction (Turner and Turner, 1980). The reduction in G6P/F6P ratio relative to harvest in asparagus spears tips may, therefore, indicate that the enzymatic status of PGI was altered. In addition, O_2 levels below 1 kPa caused accumulation of fermentation products ethanol and lactate (Silva et al., 1998a). Anoxia stimulates glycolysis, at least in part, via

the reaction that converts F6P to $F1,6P_2$ (Ap Rees et al., 1985). The increase in the $F2,6P_2$/F6P ratio at O_2 partial pressures below 1 kPa supports a role for regulation of fermentative carbon flux at this step.

The unchanged amounts of GAP relative to DHAP throughout the all O_2 range and CO_2 treatments may indicate that the equilibrium for triose phosphate isomerase (TPI, EC 5.3.1.1) was not altered by O_2 and/or CO_2 treatments.

The higher 3-PGA content at the hypoxic O_2 range may suggest an inhibition of the cytosolic FBPase (Stitt, 1990), which, combined with the fact that the highest activity of PPi:PFK in the tip is relatively higher than ATP:PFK, suggests carbon flux at that point was directed toward glycolysis. Barker et al. (1966) discussing the Pasteur effect, generated by anoxia in pea seedlings, attributed a decrease in the levels of 3-PGA and PEP to higher rates of glycolysis. They suggested that those changes reflected an acceleration of the glycolytic flux resulting in formation of fermentation products as a short term alternative for the cell resupply/keep its energy. As a result of anoxia, the contents of, G6P, F6P, and pyruvate increase (Scott et al., 1995). In this experiment, an increase in PEP at range of 2 to 4 kPa O_2 was detected, which was followed by a marked decrease at O_2 below 1 kPa (Fig. 3.6) for all CO_2 levels. The pattern for PYR was converse to that for PEP, showing an increase in PYR contents when contents of PEP were lower (below 1 kPa O_2). This data is also reflected in the crossover plots and suggests that glycolysis was regulated at that step for asparagus spear tips experiencing limiting levels of O_2.

Glycolytic enzymes. HK are enzymes recognized to be involved in short-term acute regulation of glycolysis (Meyrick, 1990), possibly acting as the sugar sensor in

higher plants (Jang et al., 1997), by transducing the signal that connects glycolysis with photosynthesis (Jang and Sheen, 1997). HK are also the enzymes that metabolize most of the glucose and fructose produced by the hydrolysis of sucrose, which is sent forward throughout glycolysis. The level of 20 kPa CO_2 caused a reduction in HK activity. The reduced activity of HK is consistent with the lower contents of G6P and F6P, and by the reduced sucrose content found in the same CO_2 level. Bouny and Saglio (1996) found that under anaerobiosis, phosphorylation of hexoses by HK was the major limiting step in glycolysis when the pH of the cytoplasm dropped to around 6.5. The decreased glycolytic flux was attributed not to an increase in proteolytic activity but to the reduction in the levels of ATP and pH. Low pH acts in the cell as a fine metabolic control, which modulates the activity of enzymes, and therefore affects the flux of metabolites through the pathway (Plaxton, 1996). As the pH was not measured in this experiment, and considering the saturated conditions in which the enzyme assays were conducted, these results also can be attributed to a change in the amount of this enzyme. Furthermore, similarly to Bouny and Saglio (1996), in asparagus spear tips ATP declined at O_2 partial pressure below 1 kPa (Silva et al., 1998b), which supports the reduced hexokinase activity observed at similar O_2 levels.

FK phosphorylates fructose specifically (Turner et al., 1977b) as the initial step of glycolysis, following the split of sucrose into glucose and fructose. In immature tomato fruit (Schaffer and Petreteikov, 1997), FK is reported to be the one of the key enzymes for sucrose metabolism, in an apparently coordinated manner with SS. In asparagus tips, much of thee incoming carbon is metabolized to cell walls. The UDP-glucose formed by

SS is rapidly consumed for cell wall formation (Huber and Akazawa, 1986). The higher FK activity found at harvest, may indicate that was a need to metabolize fructose at that time, and sucrose was metabolized mostly via SS. However, conversely to HK, FK activity was reduced more than half at the steady state O_2.

G6PDH is the enzyme that catalyzes the first reaction from the pentose phosphate pathway, which primary function is thought to be supplying NADPH to the cytoplasm for use in biosynthetic processes (Turner and Turner, 1975). In this way, elevated activity of this enzyme is associated with tissues that possess high biosynthetic activity (Plaxton, 1996). Changing the levels of CO_2 and O_2 may result in changes in the amount of enzymes and/or expression of different genes for the regulation of biosynthetic processes. The data from this experiment shows that most of the changes in metabolite pools and enzyme activities was detected below 2 kPa O_2. This may indicate that changes in the expression of different genes for *de novo* synthesis of several isozymes may be occurring around this O_2 level. This implies a large requirement for reducing power to supply demanding biosynthetic processes for growth and protein synthesis, which may explain the high G6PDH activity in asparagus tips and the sudden increase in activity below 2 kPa O_2.

Low O_2 partial pressure (below 2 kPa O_2) caused a reduction in the content of both F6P and F1,6P_2, relative to the harvest, which appeared to be independent of CO_2 (Fig. 3.2). Low O_2 caused elevated levels of F1,6P_2 relative to F6P, which is interpreted to indicate a promotion of glycolysis by one or more of the two enzymes catalyzing this interconversion: ATP-PFK and PPi-PFK. However, in asparagus spears tips, only PPi:PFK underwent an increase in activity below 2 kPa O_2. High CO_2 concentrations

affected levels of intermediate that have regulatory roles. Plant ATP-PFK, for instance, can be inhibited by ATP and PEP (Turner and Turner, 1980). In pea seed, for example, ATP:PFK is inhibited by PEP (Kelly and Turner, 1970). This inhibition is relieved by F6P, $MgCl_2$, and Pi. However, ATP:PFK is not regulated by the simple adenylate energy charge, but, although it requires ATP as substrate, it is inhibited by high concentrations of ATP (Dennis and Greyson, 1987). The principal regulation of plant ATP:PFK appears to be by the PEP/Pi ratio. However, metabolites closely related to PEP, 3-phosphoglycerate and 2-phosphoglycerate also inhibit the enzyme (Dennis and Greyson, 1987). In carrots, ATP:PFK enhances the flux of metabolites through the glycolytic pathway, which is associated with an increase in respiration (Adams and Rowan, 1970). Alternatively, regulation of glycolysis by ATP-PFK, which functions strictly in the glycolytic direction, depends to a great extent on the pH (Turner and Turner, 1980). High CO_2 results in a slight reduction in pH (Hess et al., 1993) and decline of the activity of both ATP- and PPi-PFK (Kerbel et al., 1988), as also demonstrated in this experiment by the crossover plot (Figs. 3.7 and 3.8). In this work, pH was not measured, however, a sudden increase in PEP at 2 to 4 kPa O_2 (Fig. 3.6) coincided with a concomitant reduction in the ATP:PFK activity. From 2 to 16 kPa O_2, ATP:PFK activity was higher as CO_2 levels decreased, suggesting that CO_2 caused an inhibition on its activity. In asparagus tips, ATP was found to decrease as O_2 decreased (Silva et al., 1998b), that is consistent with similar decrease in ATP:PFK activity. It can be that, as O_2 decreased, the decline in ATP resulted in the displacement of the ATP:PFK reaction from equilibrium and accumulation of $F2,6P_2$ relative to F6P, slowing glycolytic flux in asparagus spear tips.

PPi:PFK is a near-equilibrium reaction and could in principle, catalyze a net flux of carbon in the direction of glycolysis or gluconeogenesis, and is involved in the regulation of cytosolic PPi (Stitt, 1990). However, it may catalyzes a reaction that is directed toward glycolysis primarily in tissues dominated by biosynthesis and the use of respiratory intermediates for the synthesis of cellular material (Ap Rees et al., 1985) as occur is asparagus spear tips. ATP:PFK is involved in glycolysis when metabolites for mitochondrial energy metabolism are required, while PPi:PFK activity exceeds ATP:PFK activity in tissues engaged in biosynthetic activity (Dennis and Greyson, 1987). The high metabolic rate reported for asparagus explains the higher PPi:PFK activity relative to ATP:PFK found in spear tips at harvest. The enhancement of PPi:PFK activity below 2 kPa O_2 was probably due to an enhancement of the glycolytic flux, and a possible increase in protein synthesis. Below 2 kPa O_2, PEP content decreased while an increase in PYR content relative to harvest was detected for all CO_2 treatments (Fig. 3.6). This decrease in PEP might, therefore, be also resulted in increased glycolytic carbon flux through a stimulation of the F6P/F1,6P_2 interconversion step via regulation of ATP-PFK, which is strongly inhibited by micromolar quantities of PEP (Turner and Turner, 1980). Furthermore, assuming the rate of photosynthesis and sucrose synthesis were minimal under the conditions this experiment was performed, the increase in F6P (Fig. 3.2) and PPi and lower Pi, in range of O_2 partial pressure greater than 2 kPa (Silva et al., 1998b) would favor reaction of PPi:PFK toward glycolysis (Stitt, 1990; Theodorou and Plaxton, 1996). In *Brassica nigra*, a large induction of PPi:PFK activity appear to be based on *de novo* synthesis of the α subunit of the enzyme, leading to a significant enhancement in activation

by $F2,6P_2$ (Theodorou, et al., 1992). $F2,6P_2$ levels were not measured in this work, however, the increase in PPi:PFK activity as the level of O_2 decreased below 2 kPa supports the contention that glycolysis was promoted as O_2 became limiting.

The crossover plot comparing 0 kPa CO_2 with higher CO_2 treatments (Fig. 3.8 A, B, and C) indicate that the conversion of F6P to $F1,6P_2$ was the step more affected by CO_2, mainly for O_2 levels above 1 kPa. However, a slight enhancement by CO_2 on the conversion of PEP to PYR, and thus on PK, was also detected, mainly for O_2 below 9 kPa. Furthermore, Fig. 3.8 also suggests that the conversion of $F1,6P_2$ to triose-P by aldolase (EC 4.1.2.13) also was affected by 5 kPa CO_2 at the lowest O_2 level achieved.

The increase in PK activity below 2 kPa O_2 was coincident with a similar increase in PYR content in the same O_2 range (Fig. 3.6). An increase in PYR levels with a simultaneous decrease in PEP (i.e. an increase in the PYR/PEP) also indicates a promotion of the conversion of PEP to PYR, which is consistent with the increase in PK activity (Fig. 3.10).

The crossover plot comparing the highest O_2 (16 kPa) with lower O_2 levels for each CO_2 treatment (Fig. 3.9 A, B, C, and D) indicates that the conversion of PEP to PYR (PK) was the step most affected by low O_2. PK appears to be much affected as CO_2 level decreases, suggesting that high CO_2 may cause an unbalance in the system.

In summary, O_2 levels below 1 kPa caused a reduction in all glycolytic intermediates tested, a slight increase in the ratio G6P/F6P, and an increase in the ratios of $F1,6P_2$/F6P and PYR/PEP, whereas storage at O_2 levels above 1 kPa led to a decrease in the ratio of $F1,6P_2$/F6P. 20 kPa CO_2 caused a reduction in sucrose content, a reduction

in the G6P/F6P ratio, and, when combined to low O_2, an increase in the ratio PYR/PEP. At O_2 partial pressures below 2 kPa, the decline in PEP and increase in PYR indicates a high level of control exerted at this conversion. The decline in sucrose was most affected by 20 kPa CO_2, which is supported by the higher activities of sucrose synthase and acid invertases. In contrast, 5 kPa CO_2, resulted in the least amount of sucrose loss (Silva et al., 1998a) and had resulted in the lowest SS and invertase activities, suggesting control over sucrose loss by these enzymes. The disappearance of this respiratory substrate at lower O_2 levels was paralleled by an enhancement of the conversion of PEP to PYR. This suggests elevated CO_2 may enhance glycolysis with a resulting loss in carbon from the sugar pool.

Collectively, the data suggest carbon flux in asparagus spears stored under low O_2 /high CO_2 are markedly affected by the levels of CO_2 present in the environment. CO_2 exert a strong effect on the activities of ATP:PFK, PPi:PFK and PK, enzymes known by its regulatory role in glycolysis (Plaxton, 1996). Although the effects of low O_2 /high CO_2 seems to be interactive, the results also suggest clearly separated effects of each gas. Low O_2 seems to have a more pronounced action on PK, end of the glycolytic pathway, causing acceleration of glycolysis, promoting the shift from oxidative respiration to fermentation resulting in the activation of PDC and accumulation of fermentative products (Silva et al., 1998a). On the other hand, high CO_2, besides having an effect on PK, also appeared to have a larger effect on the conversion of F6P to F1,$6P_2$, via effects on ATP:PFK and PPi:PFK activities, which strongly suggest an induced promotion of glycolysis.

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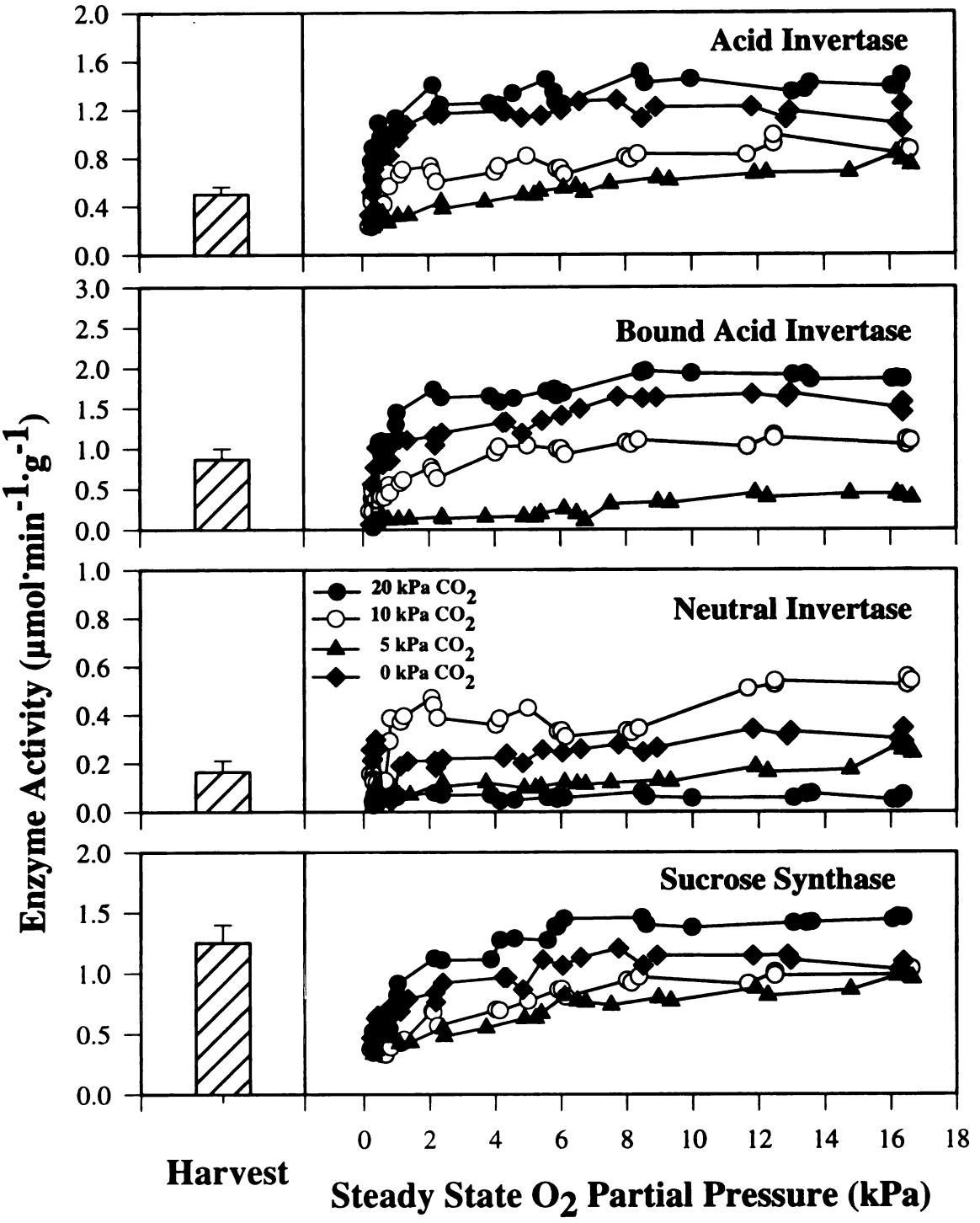

Figure 3.1. The effects of storage O_2 and CO_2 on the activity of acid, bound acid, neutral invertases, and sucrose synthase (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

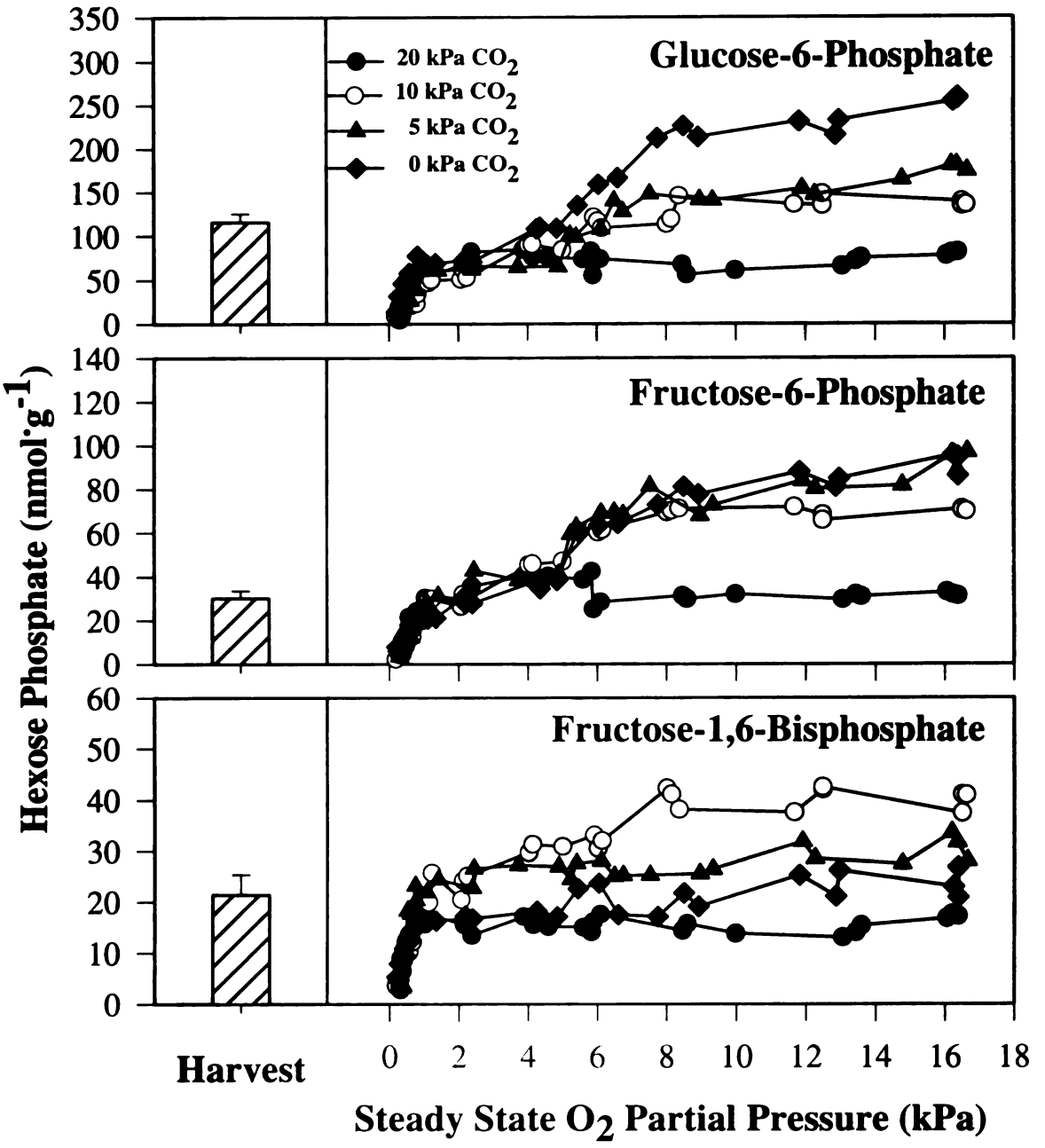

Figure 3.2. The effects of storage O_2 and CO_2 on the contents of glucose-6P, fructose-6-P, and fructose-1,6-P_2 (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

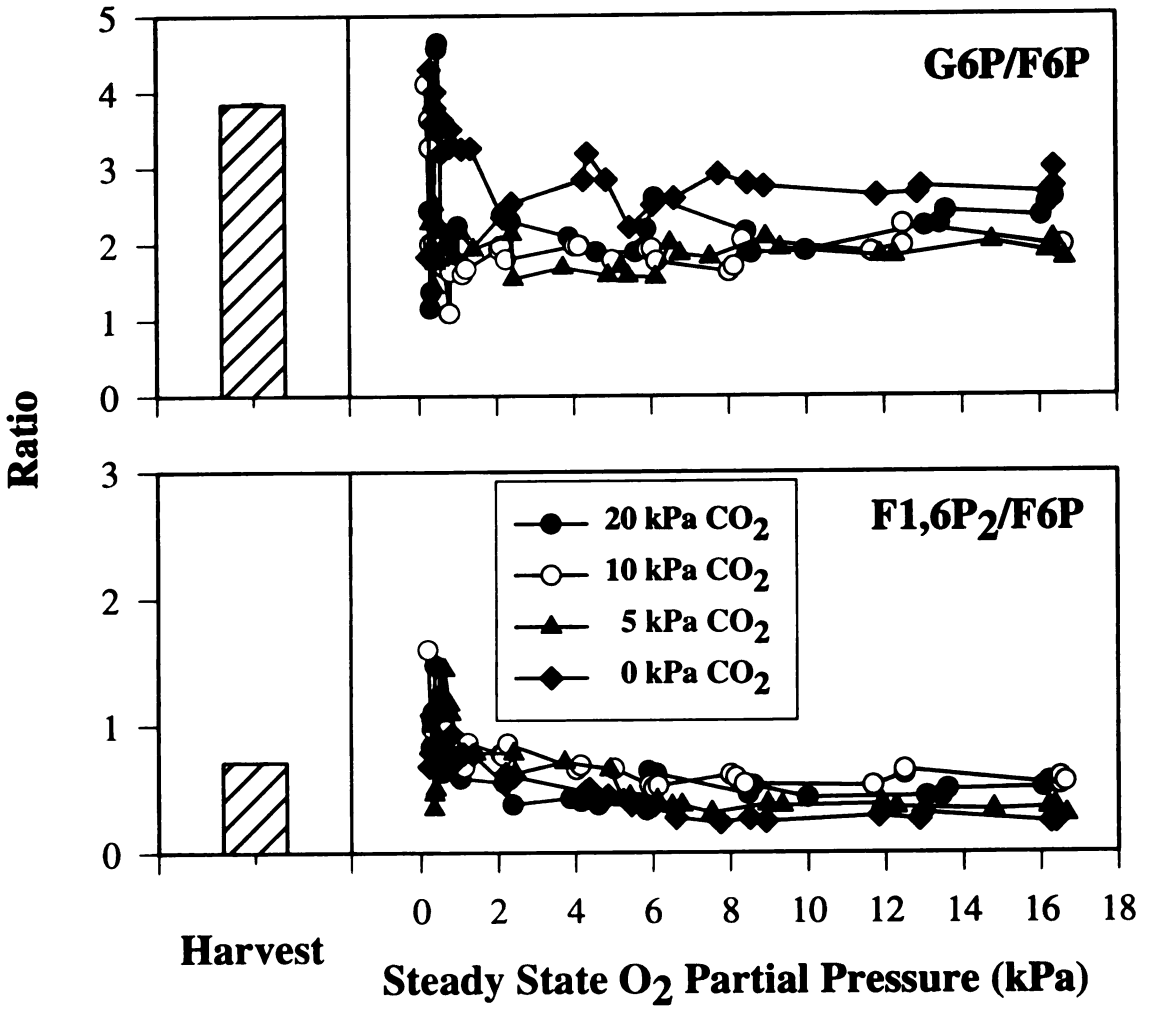

Figure 3.3. The effects of storage O_2 and CO_2 on the ratios of glucose-6-P to fructose-6-P, and fructose-1,6-P_2 to fructose-6-P (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

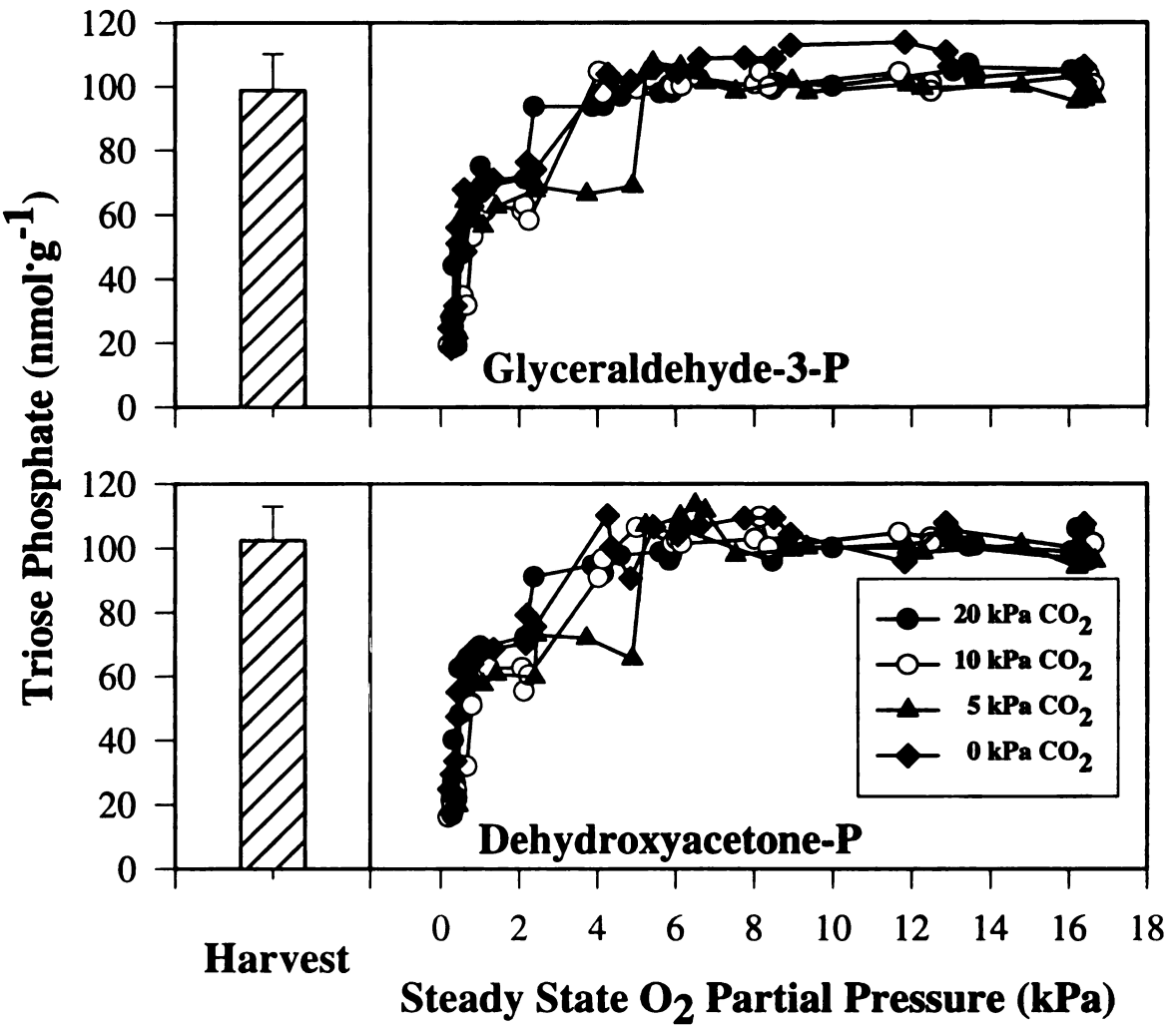

Figure 3.4. The effects of storage O_2 and CO_2 on the contents of glyceraldehyde-3-P and dehydroxyacetone-P (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

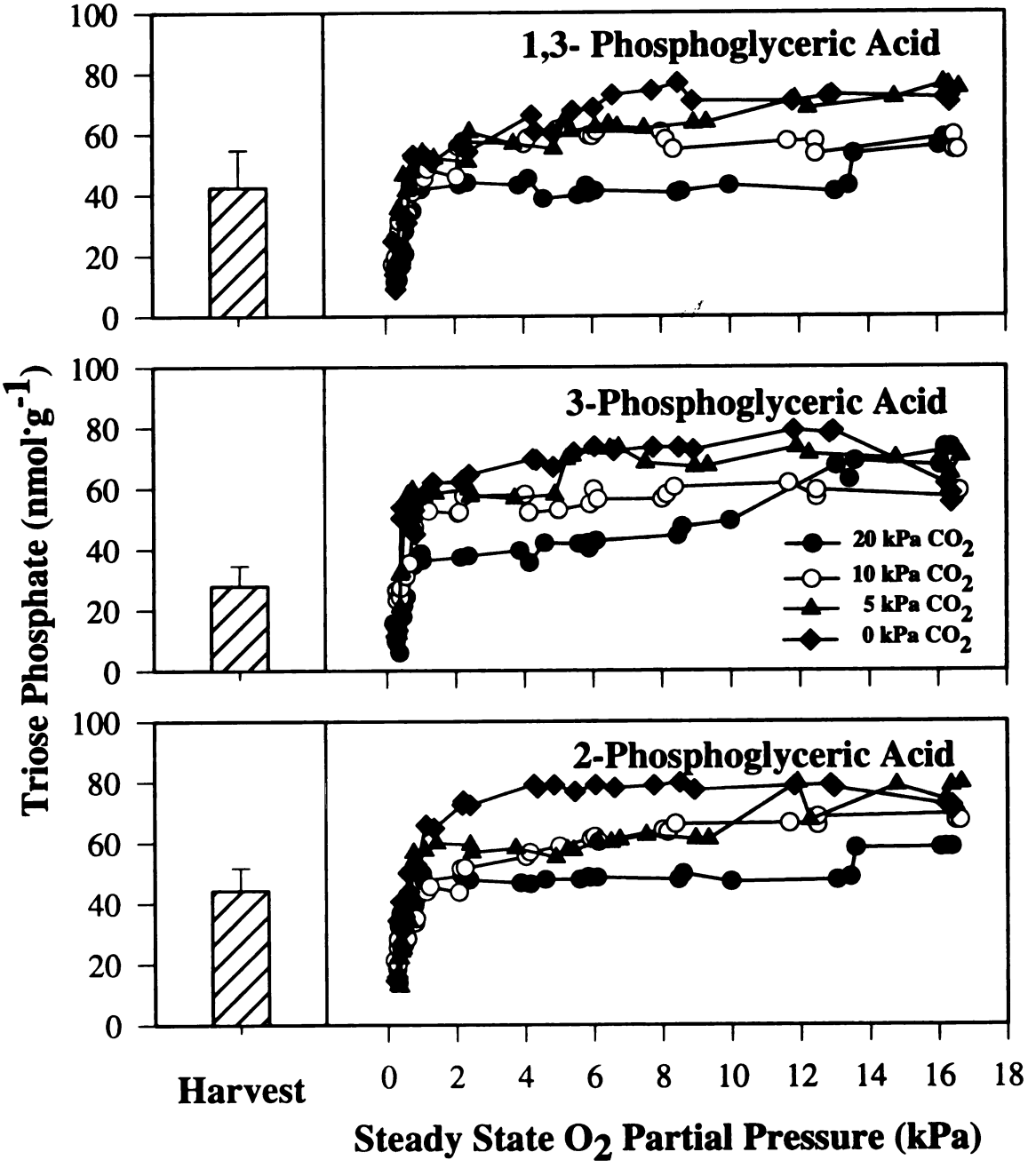

Figure 3.5. The effects of storage O_2 and CO_2 on the contents of 1,3 phosphoglyceratic, 3-phosphogliceric, and 2-phosphoglyceric acids (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

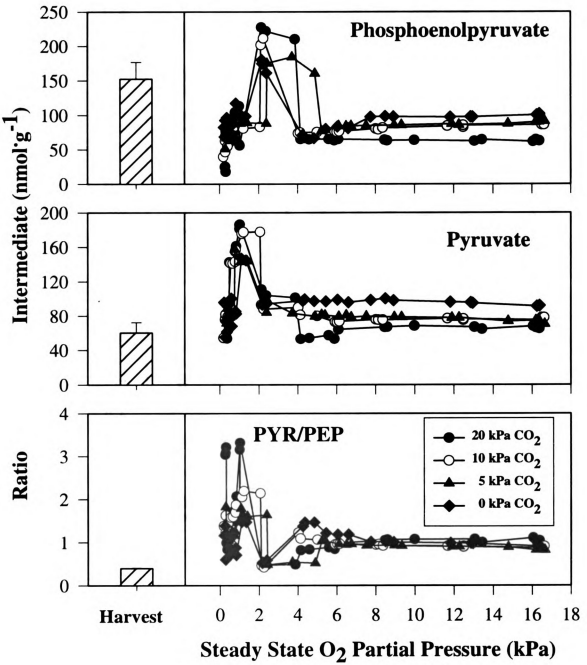

Figure 3.6. The effects of storage O_2 and CO_2 on the contents of phosphoenolpyruvate (PEP), pyruvate (PYR) (fresh mass basis), and the PYR/PEP ratio, relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

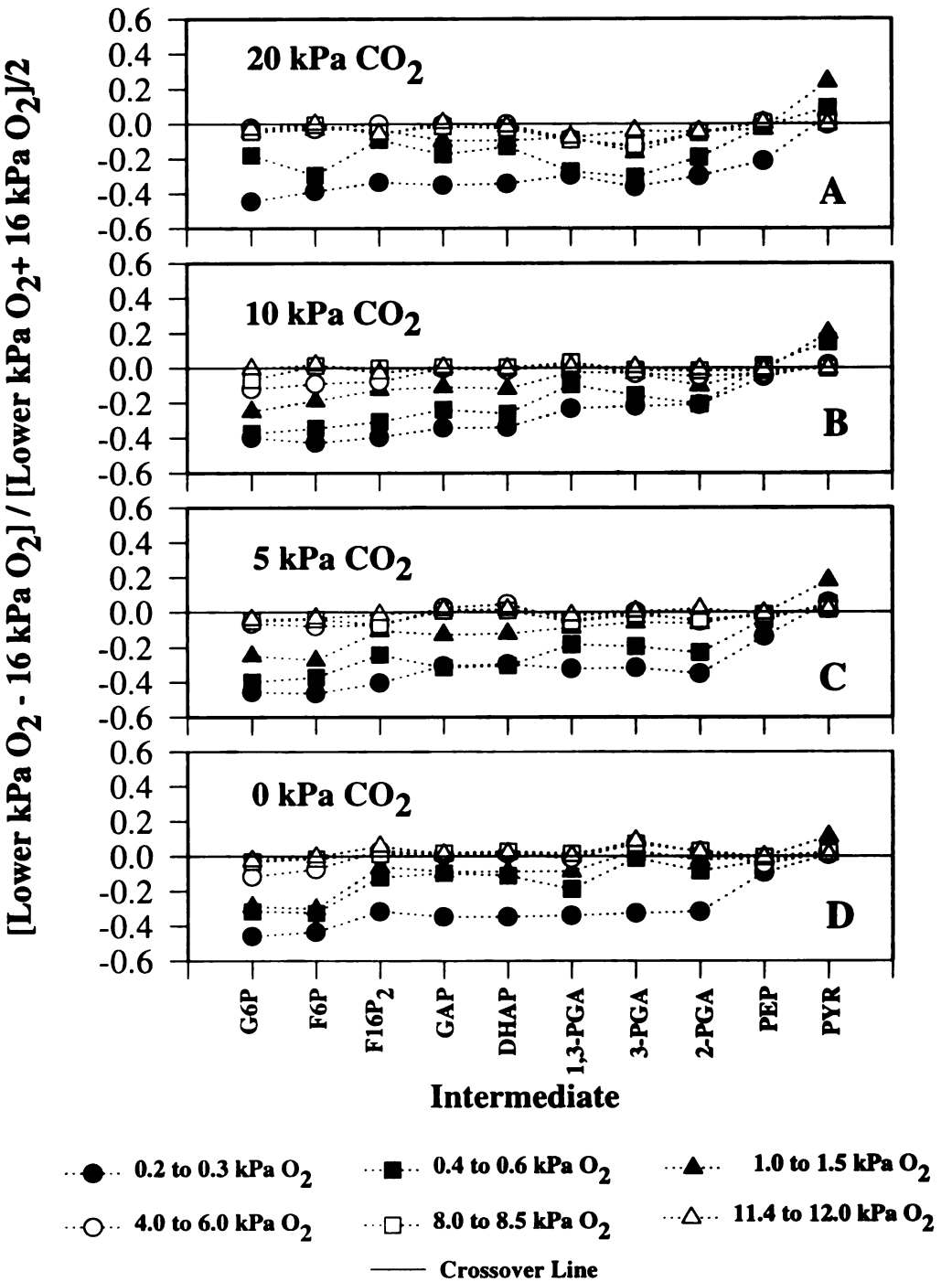

Figure 3.7. Crossover plot comparing lower O_2 levels with the highest O_2 level (16 kPa) for CO_2 levels of 20 (A), 10 (B), 5 (C), and 0 (D) kPa, in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

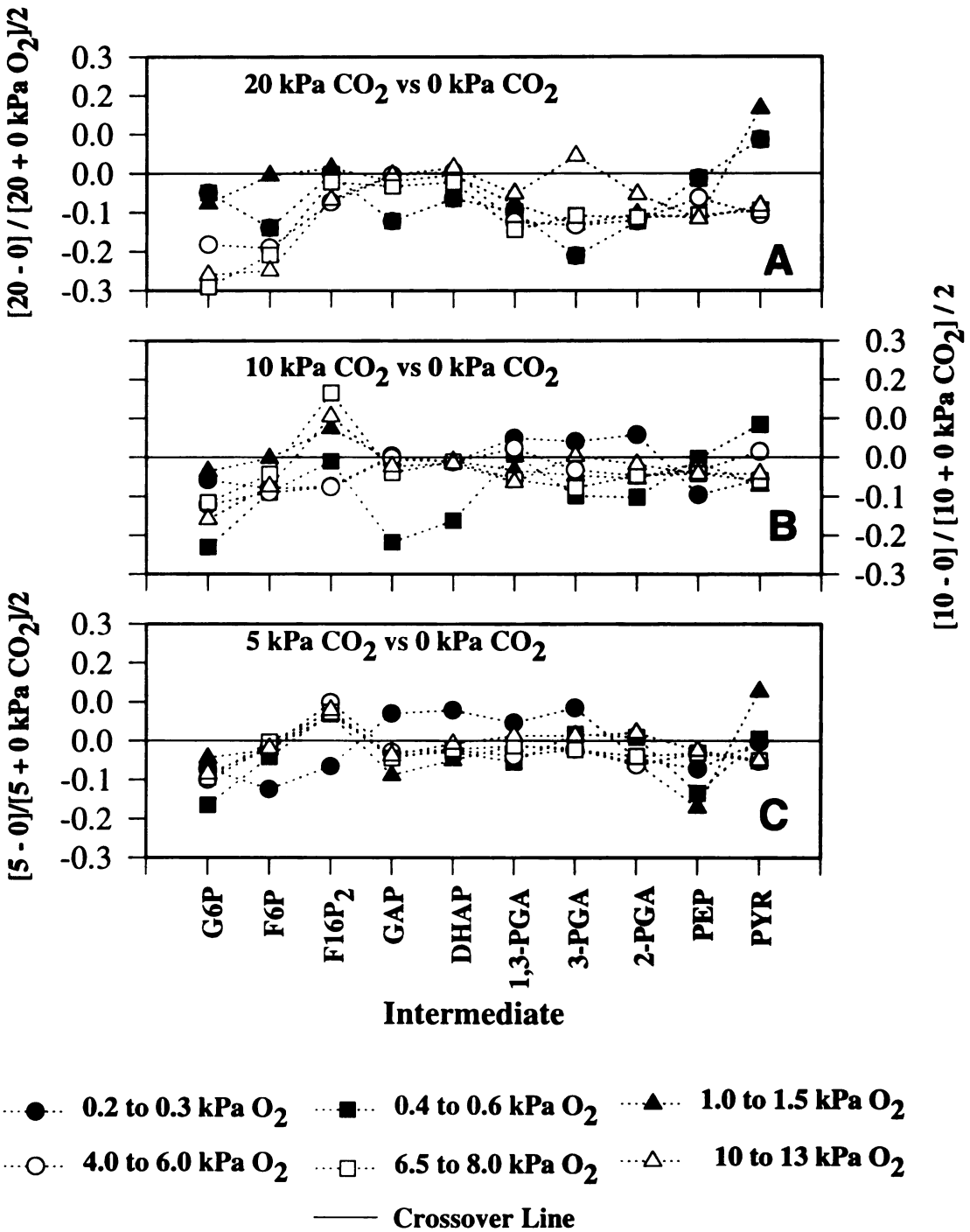

Figure 3.8. Crossover plot comparing higher CO_2 levels (20 (A), 10 (B), and 5 (C) kPa) with the lowest CO_2 level (0 kPa), in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

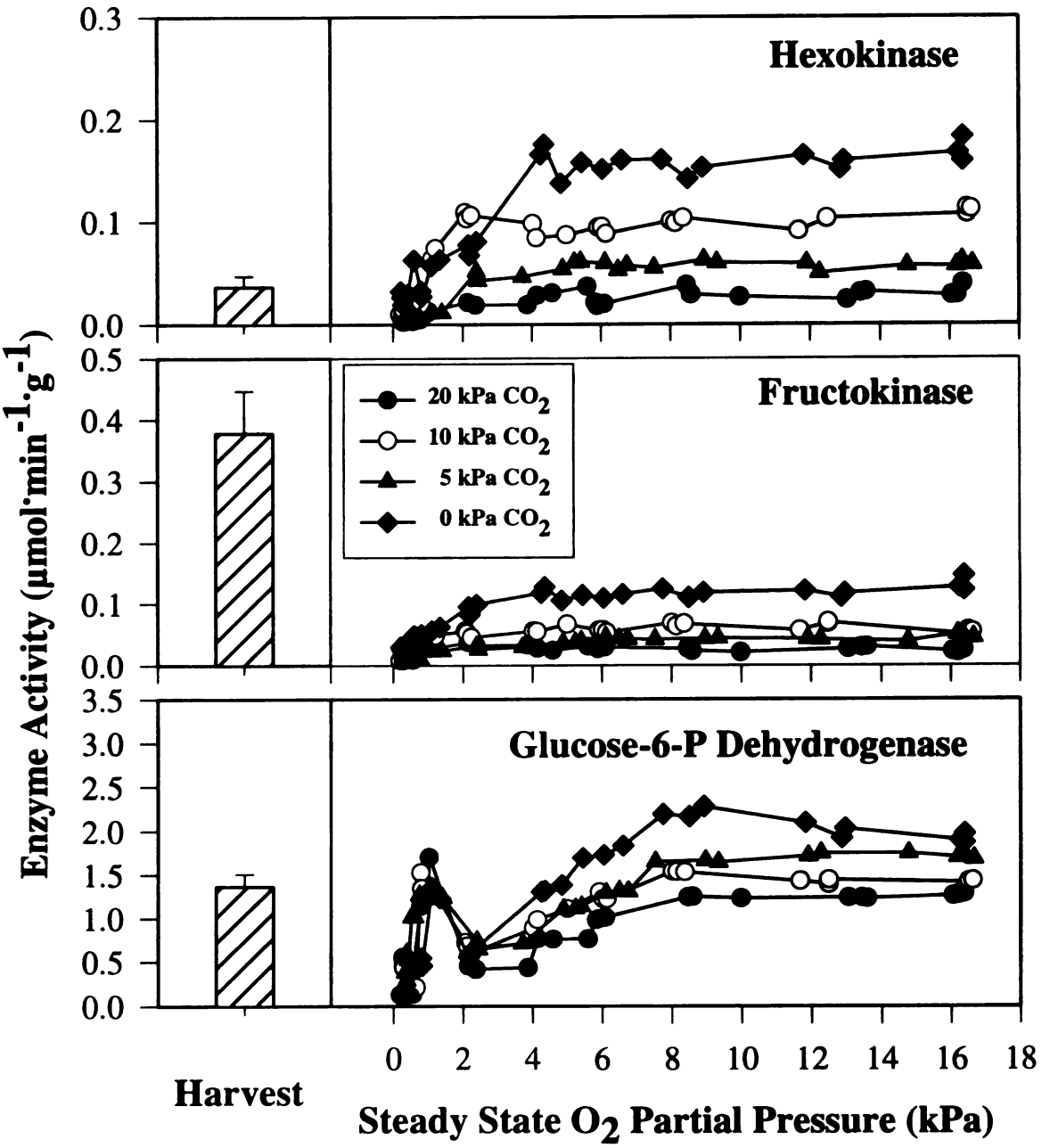

Figure 3.9. The effects of storage O_2 and CO_2 on the activities of hexokinase, fructokinase, and glucose-6-P dehydrogenase (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

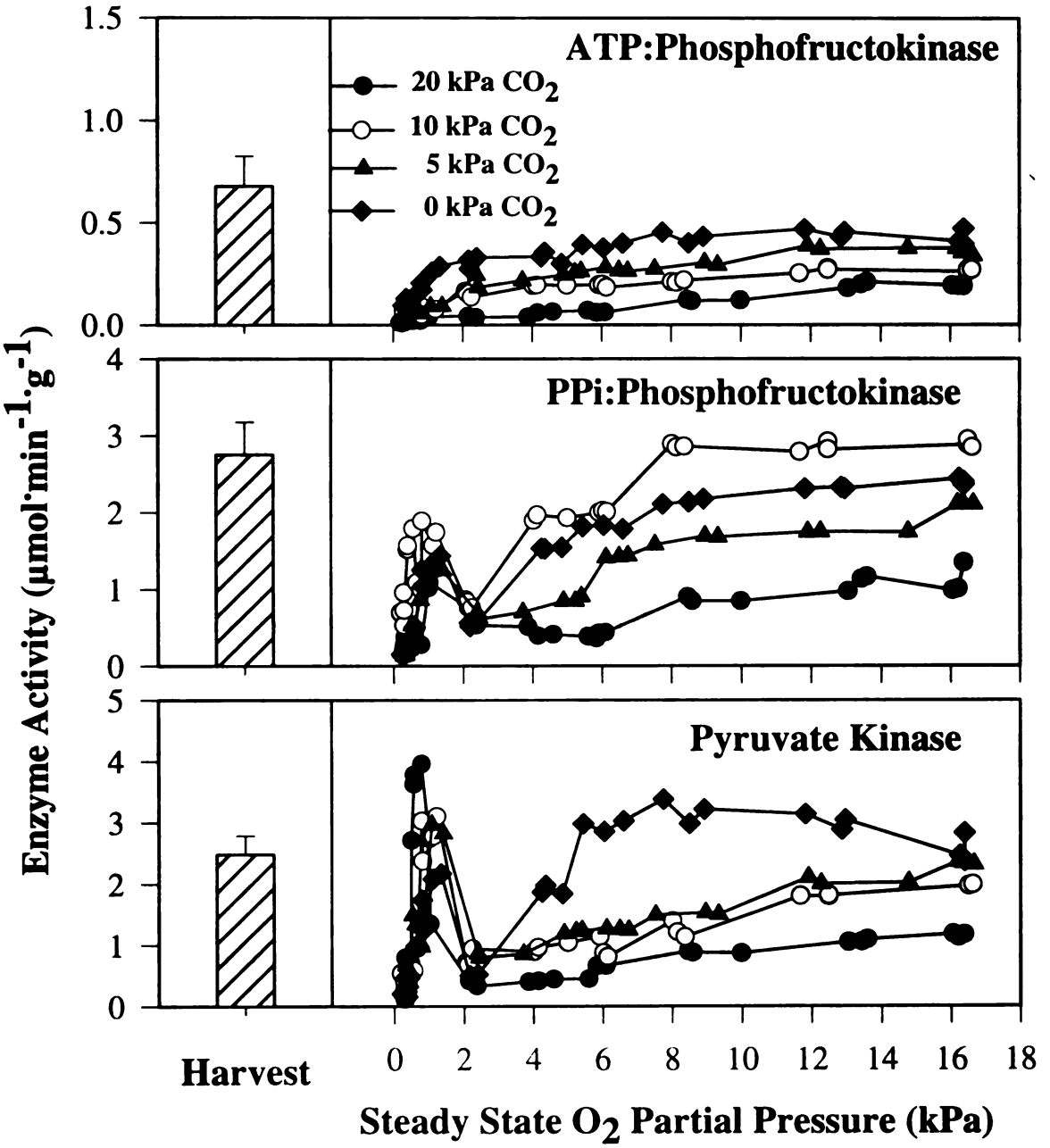

Figure 3.10. The effects of storage O_2 and CO_2 on the activities of ATP:phosphofructokinase, PPi:phosphofructokinase, and pyruvate kinase (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spears held at 1 °C for 6 days.

CHAPTER 4

PHOSPHATE AND NUCLEOTIDE METABOLISM UNDER LOW O_2 /HIGH CO_2 PARTIAL PRESSURES IN THE TIPS OF HARVESTED ASPARAGUS SPEARS STORED AT 1 °C

INTRODUCTION

Asparagus spears are characterized by a very high postharvest metabolic rate (King et al., 1990; Silva et al., 1998a; Silva et. al., 1997). The rate of metabolism is particularly high in the developing meristem (spear tip). Harvesting the spear removes the source of carbohydrate, leading to carbohydrate starvation. Sugar starvation triggers several physiological, biochemical, and molecular events in plants, among them: 1. the depletion of intracellular carbohydrate content (Journet et al., 1986; Saglio et al., 1980); 2. an increase in inorganic phosphate (Pi) (Journet et al., 1986); a concomitant decline in nucleotide tri-phosphate (Saglio et al., 1980); 3. senescence and deterioration, leading to loss of cell ultrastructure (King et al., 1990; Silva et al., 1998a).

Plant cells, whether photosynthesizing or not, produce enormous quantities of ATP from ADP, indicating that, *in vivo*, the utilization of ATP is extremely well adjusted to its regeneration (Pradet and Raymond, 1983; Saglio et al., 1980). In aerobic conditions, ATP is generated by the oxidative processes of glycolysis, oxidative phosphorylation, and oxidative pentose phosphate pathways (Tetlow et al., 1998). In response to low O_2, most plants experience a reduction in oxidative phosphorylation, which typically results in a marked inhibition of respiratory (i.e. O_2-linked) ATP synthesis (Raymond et al., 1985). Under conditions of limiting O_2, plants shift to a lower overall

rate of ATP synthesis via fermentation with a concomitant recycling of NAD (Rébeillé et al., 1985). As adenylate metabolism changes in response to low O_2, a shift in all the major phosphate and nucleotide pools occurs.

Low O_2 and/or high CO_2 stresses induce a number of metabolic and energetic modifications, linked to modifications of gene expression in an as yet unknown manner (Ricard et al., 1994). However, determining the relative importance of biochemical shifts in enzyme activities and metabolite pools in plant performance under stresses is complicated by the fact that stresses disturb whole networks of biochemical processes (Rébeillé et al., 1985). On the other hand, by reducing respiratory rate through low O_2 and altering metabolism by elevated CO_2 partial pressures, it may be possible to bring a commodity to a new steady metabolic activity at any level between the highest reached under aerobic conditions and the lowest level reached under hypoxia. This may allow to reach a lower metabolic rate, which may result in delaying senescence. However, research is needed to evaluate how phosphate and adenylate status interact and how those factors may be affected by respiration and carbohydrate metabolism, in terms of metabolic energy, under reduced levels of O_2/elevated CO_2 partial pressures in harvested asparagus spears.

PPi, Pi, ATP, and ADP are utilized and/or generated in a number of different steps in the glycolytic pathway (Duff et al., 1989). These metabolites are critical in satisfying the energy requirement of a tissue (Pradet and Raymond, 1983), and regulating the mobilization of stored carbohydrates (Rébeillé et al., 1985).

Pyrophosphate (PPi) is thought to serve as an energy source for glycolysis (Black et al., 1987). PPi is controlled in the cytoplasm by PPi:PFK, which produces PPi during

gluconeogenic carbon flux (Dancer and Ap Rees, 1989). Advantageous features have been found for some plants which, under energy stress due to anoxia, conserve ATP by switching to a PPi-based metabolism (Ricard et al., 1991). In agreement with that, in asparagus tips the extractable activity of PPi-dependent enzymes such as sucrose synthase and PPi-PFK (Silva et al., 1998b) was increased by O_2-deficiency. In addition, carbohydrate metabolism has been suggested to be collectively regulated by key metabolites such as F-2,6-P_2, sugar-P, adenylates, and Pi/PPi ratio (Koch, 1996).

Inorganic phosphate (Pi) not only plays a vital role in energy transfer and in metabolic regulation (Duff et al., 1989). For instance, under Pi starvation, in order to save energy, plants 'bypass' some ATP and ADP glycolytic steps in *Brassica nigra* (Duff et al., 1989) and maize seedlings (Ricard et al., 1991). Pi is also an important constituent of sugar-P and macromolecules in plants.

The adenine nucleotide content of the tissue and the adenylate energy charge (AEC) have been used as indicators of cellular metabolic status (Atkinson, 1968; Xia et al., 1995). AEC represents the relative saturation of the adenylate pools in phosphoanhydride bonds (Pradet and Raymond, 1993) and it has been proposed to reflect the availability of ATP relative to ADP and AMP for ATP-utilizing processes and, via feed back mechanisms, exerts control over respiration (Brouquisse et al., 1991).

This research work details the effects of 12 O_2 combined with 4 CO_2 partial pressures, ranging from hypoxic to active fermentation levels, on high and low energy phosphate pools in asparagus spears tips. Our objective was to understand how these processes are regulated postharvest during spears storage at 1 °C.

MATERIAL AND METHODS

Plant material. Asparagus spears (*Asparagus officinalis* L. cv. Jersey Giant) were harvested early in May from the Michigan State University Horticultural Research Center between 6:00 and 9:00 a.m. and stored at 1 °C. Samples were taken for analyses at harvest and after storage. Spears selected to represent harvest conditions were frozen in liquid N_2 in the field. Spears were harvested when they reached a minimum length of 240 mm and were trimmed to 180 mm. Only straight undamaged spears with closed bracts and no obvious symptoms of disease were used. Spears to be placed on storage were chilled with ice while still in the field and transported to the laboratory within the hour. After storage, spears were frozen in liquid N_2. After freezing, all spears were stored at -80 °C until analysis. There were three replications. Three replicate experiments were conducted in consecutive asparagus seasons (1995, 1996, 1997). Tissue samples comprised the most apical 45 mm of the spear.

Atmosphere generation. O_2 partial pressures ranging from a low of a.16 kPa to a high of 16 kPa were generated in packages for four CO_2 treatment levels (0, 5, 10, and 20 kPa). O_2 levels were obtained by sealing spears in packages composed of low density polyethylene (LDPE) of varying film thickness and placing the packages in air (0 kPa CO_2) or enclosing the packages in glass chambers with 20.7 kPa O_2 and 5, 10, and 20 kPa CO_2 (Beaudry, 1993). Chamber lids were fitted with inlet and outlet ports for purging with gas mixtures; a glass tube (1.3 cm i.d. x 2.7 cm long) was inserted through the lid and glued to a piece of electrical tape on the surface of the enclosed package with silicone sealant (Beaudry, 1993). The silicone sealant formed a septum through which gas samples could

be obtained from within the package. Chambers were continually purged with gas mixture of O_2, CO_2, and N_2 gases at 30 $mL \cdot min^{-1}$ to generate the target atmospheres within the enclosed packages. Actual CO_2 levels in the packages were slightly higher than reported values, but for clarity of presentation, are reported as treatment values. The gas composition of individual packages was monitored until steady state conditions were reached. Steady state conditions were achieved on day six of the study. O_2 and CO_2 gradients across the film were used to calculate the flux for both gases through the film using previously obtained permeability data (Lakakul, 1994). Flux data were used to calculate the rates of O_2 uptake and CO_2 production (Cameron et al., 1994).

^{31}P-NMR Analysis. To evaluate the inorganic phosphate (Pi) status in the sample, three spear tips (approximately 6 g total tissue mass) from the 1.0 kPa O_2 x 20 kPa CO_2 treatment were fitted into a 10 mm wide x 180 mm long NMR tube immediately after cutting. The tube was capped and capillary tubing was used to circulate air. NMR scanning was done at 1 °C, using a NMR spectrophotometer (General Electric Omega 500). Fifteen hundred transients were acquired using a 60 ° pulse, a 1.8 recycle time, 9806 complex time domain points, and a 15.4 Khz spectral width (Weich et al., 1989). Line broadening of 30 Hz was applied before Fourier transformation. Total acquisition time was 1h and 22 min. Chemical shift in all spectra are reported relative to 85% H_3PO_4 (as an external standard) and 0.5 M methylenediphosphonic acid (as an internal standard).

PPi and Pi and Adenylate Extractions. Approximately 3 g of frozen tissue per sample were transferred to liquid N_2 in a precooled mortar, in which 5 mL of ice-cooled 0.8 N (v/v) $HClO_4$ were added to the frozen tissue, and ground with a pestle to a fine

powder without melting. Five mL of ice-cooled 0.8 N $HClO_4$ (total 10 mL) were then added to the powder, which was extracted for 30 min at 0 °C with occasional shaking, followed by centrifugation at 30,000g for 30 min at 0 °C. Supernatant pH was adjusted to pH 7.0 to 7.2 with 5 N K_2CO_3, allowed to sit on ice for 10 min, and clarified by centrifugation at 30,000g for 10 min (Scott et al., 1995). Immediately following extraction, the resulting supernatant was used for analysis of PPi (Edwards et al., 1984), Pi (Cornell et al., 1979;), NTP (Mohanty et al., 1993; Trautchold, et al., 1985), ADP, and AMP (Jaworek and Welsch, 1985; Scott et al., 1995). To estimate metabolite losses during extraction, measured amounts of adenylates roughly equal to the amounts present in the tissue, were added to the extracting medium. Resultant recoveries were 93%, 95%, 98%, 89%, and 91%, for PPi, Pi, ATP, ADP, and AMP, respectively. The recovery assays were run in five replicates.

PPi and Pi and Adenylate Assays. PPi, Pi, ATP, ADP, and AMP were assayed at 25 °C using a spectrophotometer (Hitachi, Model U3000, Hitachi Inc., Tokio, Japan) with an attached refrigerator circulator (Polystat, Cole-Palmer Instrument Co., Chicago, IL), by measuring the absorbance of pyridine nucleotides at 340 nm following adaptations from procedures described by Kato-Noguchi and Watada (1996), Scott et al., (1995), and Trautchold, et al., (1985). Measurements were taken for 15 to 20 min in a final volume of 1mL of buffer mixed with other additions specified for each metabolite. Depending on the metabolite, 50 to 100 µL crude extract was used. Each reaction was allowed to go to completion. Blank cuvettes for each reaction contained all the reagents listed for each metabolite except that the extract was replaced with water.

Assay cocktails were as follows: PPi: 60 mM Tris-acetate (pH 8.0), 2 mM of magnesium acetate, 6 mM fructose-6-phosphate, 10 μM of fructose-2,6-bisphosphate, 0.13 mM NADH, 1.5 units aldolase (EC 4.1.2.13), 2.5 units glycerol-3-phosphate dehydrogenase (EC 5.3.1.1), 7 units triosephosphate isomerase (EC 5.3.1.1). The reaction was started by the addition of 0.3 unit PPi:PFK.

Pi: 100 mM TEA (pH 7.6), 1.5 mm EDTA, 2 mM $MgCl_2$, 1.5 mM NAD, 15 mM glucose, 1 mM ADP, 0.5 mM fructose-1,6-bisphosphate, 3 units aldolase, 8 units glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 2 units hexokinase (EC 2.7.1.1), plus 10 units phosphoglycerate kinase (EC 2.7.2.3). The reaction was started by adding the extract.

Adenosine 5'-triphosphate (ATP): 150 mM TEA (pH 7.6), 2.5 mM $MgCl_2$, 1.5 mM NADP, 2 mM glucose, 4 units glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 2 units hexokinase. The reaction was started by adding the extract.

Adenosine 5'-diphosphate and adenosine 5'-monophosphate (ADP and AMP, Jaworek and Welsch, 1985): 100 mM TEA (pH 7.6), 1 mM phosphoenolpyruvate, 30 mM $MgCl_2$, 0.12 mM KCl, 0.3 mM NADH, 20 units lactate dehydrogenase (EC 2.7.4.3), 18 units pyruvate kinase (EC 2.7.1.40) for ADP measurement; the above mixture for ATP plus 16 units myokinase (EC 2.7.4.3) for AMP measurement.

Adenylate Energy Charge (AEC). The AEC was expressed according to Atkinson (1968) as:

$$AEC = ([ATP] + 0.5 * [ADP]) \cdot ([ATP] + [ADP] + [AMP])^{-1} \quad [1].$$

RESULTS

PPi. In asparagus tips at the steady state, PPi content at 0 kPa CO_2 treatment and 16 kPa O_2 decreased relative to harvest. The PPi content for the 5 kPa CO_2 treatment tended to be higher than 0 and other CO_2 treatments at O_2 partial pressures greater than 6 kPa. Within the same O_2 range, CO_2 partial pressures of 10 and 20 kPa markedly reduced PPi content. Between 2 and 16 kPa O_2, PPi decreased as CO_2 increased and O_2 decreased (Fig. 4.1). Relative to harvest, however, the 20 kPa CO_2 treatment resulted in a decline in PPi of approximately 50 %. O_2 levels below 2 kPa, resulted in a large decrease in PPi (more than 90 %) in a manner that appeared to be independent of CO_2 treatment.

Pi. The ^{31}P-NMR inorganic phosphate profile after 6 days at 20 kPa CO_2 and 1 kPa O_2 indicated of the amounts of Pi present in the vacuole was very high relative to that present in the cytoplasm (Fig. 4.2). The Pi content in the vacuole was significantly higher than the nucleotide phosphate pools present in the cytoplasm.

The Pi content at 0 kPa CO_2 and 16 kPa O_2 was slightly lower than at harvest (Fig. 4.3). However, although Pi at 0 and 5 kPa CO_2 were maintained close to harvest content at 16 kPa O_2, Pi increased for 10 and 20 kPa CO_2. Pi tended to increase as O_2 partial pressure declined. O_2 levels below 2 kPa caused a substantial increase in Pi content. Below 2 kPa O_2, Pi enhancement appeared to be dependent on increased CO_2 levels, since Pi content was enhanced about seven times for 20 kPa CO_2 treatment. The PPi/Pi ratio declined with increasing CO_2 and decreasing O_2 (Fig. 4.3, insert).

Adenylate. Relative to harvest, ATP content decreased approximately 20% for 0 kPa CO_2 and 16 kPa O_2 (Fig. 4.4). Although ATP values at 0 and 5 kPa CO_2 were close

at 16 kPa O_2, for O_2 levels below 16 kPa O_2 and above 6 kPa O_2, ATP tended to be higher for 5 kPa CO_2. At 16 kPa O_2, CO_2 partial pressures above 5 kPa CO_2 caused ATP to decrease. From 2 to 16 kPa O_2 ATP content was the lowest for 20 kPa CO_2 treatment. Between 6 and 16 kPa O_2, decreasing O_2 caused a decline in ATP only for the 20 kPa CO_2 treatment. However, ATP appeared to be affected by 20 kPa CO_2 treatment at approximately 14 kPa O_2 (Fig. 4.4), when a marked drop in ATP occurred. Below 6 kPa O_2, however, ATP content decreased markedly for all CO_2 treatments. At the lowest O_2 levels achieved, ATP was reduced approximately 90% relative to the highest O_2 level for each CO_2 treatment. Relative to ADP and AMP, ATP was the dominant adenine nucleotide at harvest (compare Figs. 4.4 with 4.5 and 4.6). The PPi/ATP ratio was maintained around 0.4 between 2 and 16 kPa O_2. However, PPi/ATP ratio started to increase as O_2 decreased below 2 kPa O_2, peaking near 1 kPa O_2 and declining again at lower O_2 levels. In response to reduced O_2 partial pressure, respiration decreased (Silva et al., 1998a). ATP declined as respiration declined in response to limiting O_2 partial pressures. For a given rate of respiration, ATP content was reduced by CO_2 partial pressures of 10 and 20 kPa.

At 0 kPa CO_2 and at the steady state 16 kPa O_2, both ADP and AMP contents were close to those found at harvest (Figs. 4.5 and 4.6). At 16 kPa O_2, ADP and AMP contents increased, as CO_2 increased, in pattern similar to Pi, with the lowest ADP and AMP contents for the 0 kPa CO_2 treatment. The effect of decreasing O_2 partial pressure was accentuated for O_2 levels below 2 kPa. Between 6 and 16 kPa O_2, O_2 had no apparent effect on ADP content, however, ADP tended to increase with decreasing O_2 for

5, 10, and 20 kPa CO_2. AMP tended to increase with decreasing O_2 for all CO_2 treatments. This indicates that CO_2 had a marked influence on ADP and AMP accumulation when the steady state O_2 was reached.

At 0 kPa CO_2 and 16 kPa O_2, AEC was maintained close to that at harvest (Fig. 4. 7). At 16 kPa O_2 AEC declined with increasing CO_2 partial pressure. In the range of 2 to 16 kPa O_2, AEC was dependent both on CO_2 and O_2 levels, decreasing as CO_2 increased and as O_2 decreased (Fig. 4.7). Below 2 kPa O_2, AEC declined markedly with decreasing O_2 partial pressure, dropping as low as 0.32. which may reflect some physical minimal equilibrium between ADP/AMP at that O_2 range.

The ATP/ADP ratio for the 0 kPa CO_2 treatment and at the steady state 16 kPa O_2 was similar to that at harvest (Fig. 4.8). The ATP/ADP ratio decreased as CO_2 increased and O_2 decreased. For the 20 kPa CO_2 treatment, at 16 kPa O_2, there was a reduction of approximately 80 % in the ATP/ADP ratio. At the lowest O_2 partial pressures, the ATP/ADP ratio decreased more than 95%, relative to the highest O_2 partial pressures for each CO_2 treatment.

A plot of ATP/ADP ratio against AEC showed that, for the highest AEC measured (approximately 0.86), ATP/ADP was similar to harvest for 0 kPa CO_2 treatment (Fig. 4.9). ATP/ADP ratio was decreased as AEC values decreased and as CO_2 increased. The difference between CO_2 treatments was no longer obvious as AEC declined below 0.7. Interestingly, 0 kPa CO_2 maintained, at higher AEC, the highest ATP/ADP, suggesting greater Pi flux per ATP, which indicates much higher ATP regeneration.

DISCUSSION

PPi. PPi is present in the cell as a byproduct of the nucleotide-dependent reactions and is primarily associated with biosynthesis of nucleic acids and proteins (Taiz, 1986). PPi may be produced or removed by PPi:PFK, which can cycle with either ATP:PFK or Fru1,6Pase catalyzed reactions. PPi is removed in glycolysis for the production of $F1,6P_2$ and is produced in gluconeogenesis, by Fru1,6Pase, for sucrose production (Dancer et al., 1990). However, under O_2-deprivation, synthetic processes, such as cell wall and protein biosynthesis are greatly reduced (Xia et al., 1995). One would expect, therefore, a concomitant reduction in the rate of PPi biosynthesis relative to its utilization, such that the PPi pools declines. The data in the present study are consistent with this possibility. Further, the decrease in PPi with increasing CO_2 suggests that elevated CO_2 can have a similar effect to low O_2 on synthetic processes.

PPi is known as a potential energy donor in the cytosol of plant cells (Dancer et al., 1990). The marked increase in the PPi/ATP ratio at decreasing O_2 partial pressures below 2 kPa O_2, suggests the predominance of PPi- over ATP-utilizing reactions. In plants subject to anoxia, the PPi-dependent proton pump activity in tonoplast vesicles is greatly increased and accompanies an increasing capacity for proton transport, hydrolyzing PPi in the cytoplasm and pumping H^+ inside the vacuole (Carystinos et al., 1995). A drop in pH might therefore accompany the suggested increased PPi utilization relative to ATP below 2 kPa O_2. Carystinos et al. (1995) reported that, as an additional adaptation to energy stress, glycolytic enzymes reactions consuming ATP can be at least partially replaced by reactions utilizing PPi as an energy source. This idea is also supported here by

the increase in PPi/ATP ratio below 2 kPa O_2, and parallel increases in PPi:PFK and a decrease in ATP:PFK activities (Silva et al., 1998b) within this O_2 range. PPi is recognized as a source of energy in plant cells in which PPi: PFK is acting as a glycolytic enzyme (Dancer and Ap Rees, 1989; Dancer et al., 1990), which would be expected to result in a decrease in PPi content, as reported in here below 2 kPa O_2 (Fig 4.2). Saglio et al. (1980) reported that PPi:PFK seemed not to be important in driving reactions under anoxic conditions in acclimated maize root tips. However, in nonacclimated maize root tips, the activity of PPi:PFK was much higher when compared to hypoxic acclimated ones. In contrast with these authors, in asparagus spear tips that were at steady-state with respect to respiration, PPi:PFK activity increased significantly below 2 kPa O_2 (Silva et al., 1998b), without any concomitant increases in PPi, suggesting that PPi:PFK may play a similar role to alcohol dehydrogenase, which does not limit the capacity of energy production under anoxia (Xia et al., 1995).

Pi. In asparagus tips at 20 kPa CO_2 and 1 kPa O_2, Pi was found mostly in the vacuole, probably due to the limited interchange of Pi between cytoplasm and plastids under those conditions. *In vivo*, in the light plastidic Pi is known to counter-exchange with imported hexose phosphate (Tetlow et al., 1998). However, in this study, harvested asparagus spears were stored in dark at 1 °C, which meant that no significant counter-exchange of Pi occurred.

Pi is a potent feedback inhibitor of PPi:PFK in the glycolytic direction (Dancer et al., 1990). O_2 levels below 1 kPa reduced PPi:PFK activity in asparagus spear tips (Silva et al., 1998b) when the highest content of inorganic phosphate was measured for all CO_2

treatments (Fig. 4.4). This Pi increase below 2 kPa O_2 probably is due associated declines in phosphorylated organic acids and sugar-phosphates (i.e., glycolytic intermediates) (Silva et al., 1998b), and tri phosphate nucleotides (Saglio et al., 1980), protein and phospholipids (Brouquisse et al., 1991), and nucleic acids (Duff et al., 1989), and loss of selective permeability of membranes as suggested by Brouquisse et al., (1991), as well as adenylate di- and tri-phosphate hydrolysis. In sycamore cells, under sucrose starvation, the cytoplasmic-phosphorylated compounds decrease whereas intracellular Pi increases symmetrically (Rébeillé et al., 1985). In asparagus spear tips, O_2 levels below 2 kPa caused a decline in sucrose content (Silva et al., 1998a). Pi increases in the vacuole were also correlated to limited sucrose availability and a decline in triphosphate nucleotides (Roby et al., 1987). The similar responses in asparagus tips below 2 kPa O_2 may be associated either with the observed decline in sucrose or, more likely, a lack in ability to synthesize ATP. Furthermore, at 16 kPa O_2, Pi content increasing as CO_2 increased suggests that, at O_2 levels close to ambient, Pi increase may be a CO_2-dependent process.

Adenylate. Hypoxic treatment cause a decrease in ATP levels in maize root tips with a concomitant rise in ADP and AMP (Xia and Saglio, 1990), in a manner similar to that reported here as O_2 partial pressures decreased (Figs. 4.4, 4.5, and 4.6). The responses of PPi and Pi to low O_2 were consistent with the drop in ATP and increase in ADP and AMP below 2 kPa O_2. The increase in the respiratory quotient at these O_2 tensions (Silva et al., 1998a) suggests that ATP biosynthesis was compromised. Our data are consistent with this possibility. In asparagus tips, one possible explanation for the reduced ATP content at higher CO_2 treatments, even for higher O_2 levels, may be due to

an uncoupling effect on oxidative phosphorylation caused by higher CO_2 (Fanestil et al., 1963). Therefore, it seems that 20 kPa CO_2, even when combined with O_2 close to ambient (16 kPa), could be limiting for the energy supply needed for survival of asparagus spear. The decline in ATP below 6 kPa O_2 for all CO_2 treatments, showing a marked decline below 2 kPa O_2, suggests a critical threshold (approximately 0.8 kPa O_2) where the ATP seemed not to be regenerated sufficiently to sustain metabolism. This threshold appeared to coincide with similar O_2 level in which increased respiratory quotient, and lactate, acetaldehyde, and ethanol contents started to be detected in asparagus tissues (Silva et al., 1998a), and also with a marked decline in glycolytic enzyme activities (Silva et al., 1998b). These results imply a decline in the glycolytic flux below that 0.8 kPa O_2. This critical threshold for glycolytic flux under those O_2-deficient conditions may reflect the minimum rate of ATP supply necessary to sustain cell function. This decline in glycolytic flux is also supported by the low AEC values found below 1 kPa O_2, which reached 0.32 and did not decline lower. This also supports the idea that there is a critical O_2 level where the tissues start to suffer irreversible damage through degradation of vital cell components such as enzymes and nucleic acids, and accompanying a parallel increase in the activity of acid phosphatases (Roberts et al., 1984), which may result in an increased total phosphate content.

On the other hand, the observed rise in PPi:PFK, and PK activities around 2 kPa O_2, independently of CO_2 treatments, (Silva et al., 1998b) suggest an attempt by the tissue to promote glycolytic flux. The changes in these enzyme activities may indicate that a transition from hypoxic to O_2-deficient metabolism occurs below 2 kPa O_2 for asparagus,

leading to accumulation of fermentation products and deficiency in ATP resupply. As reviewed by Hochachka et al. (1996), as ATP generation by oxidative phosphorylation begins to fall off under O_2 deprivation, the energy deficit is made up by activation of anaerobic ATP supply pathways, which, as suggested by Saglio et al. (1988), upon acclimation, may result in a sustained higher glycolytic rate. Maintenance of the glycolytic flux in these acclimated tissues is argued to be due to a combination of a rise in kinase activities and decreased inhibition of regulatory enzymes resulting from a higher cytoplasmic pH and ATP content (Bouny and Saglio, 1996). Furthermore, acclimation of tissues to O_2-stress conditions results in modification of ATP-utilizing enzymes, which is measured as an increase their ATP usage through changes in combination of the amounts, catalytic turnover of these enzymes, and their affinity for ATP (Xia et al., 1995). In this experiment, it is taken into account that, after 6 d storage when the steady state O_2/ CO_2 was reached, the asparagus spears were acclimated into a steady state condition for each O_2/ CO_2 combination. Threfore, it may be that in asparagus spears tips, there was an increase in glycolytic flux around 1 kPa O_2, which was immediately followed by a decrease when O_2 levels dropped to a critical level (below 0.8 kPa).

Adenylate Energy Charge (AEC). Under energy-limiting conditions, such as O_2-deficiency, AEC is recognized as an indicator of the rate of ATP regeneration (Saglio et al., 1980). In the present study, limitation of the respiratory metabolism by low O_2 induced a drop in AEC. In maize root tips, AEC has been taken as an indicator of metabolic activity in cells where the rate of oxidative phosphorylation is O_2-limited (Saglio et al., 1988). AEC varies from 0 to 1 and ranges from 0.8 to 0.9 in health tissues. At

AEC values below 0.5, the relative activity of many ATP-regenerating enzymes is decreased, while the activity of ATP-utilizing enzymes is increased (Pradet and Raymond, 1983). AEC values around 0.9, such as those found under here (at harvest and for 0 and 5 kPa CO_2 treatments with 16 kPa O_2), characterize tissues where respiration is not limited by O_2 (Raymond et al. , 1985). However, small AEC variations at high values (0.75-0.85), as those measured in the present study for 10 and 20 kPa CO_2 at 16 kPa O_2, may correspond to significant variations in the ATP/ADP and ATP/AMP ratios, and may therefore be an indication of regulatory processes that differ between O_2 and CO_2 for enzymatic ATP-utilizing processes (Pradet and Raymond, 1983).

A high AEC value and ATP/ADP ratio indicate that ATP-regenerating pathways (glycolysis and oxidative phosphorylation) do not limit the energy supply to the metabolic processes evolved upon imposition of hypoxic stress. However, below a critical O_2 level of approximately 1 kPa glycolysis is diverted to fermentation in asparagus (Silva et al., 1998a) probably due to an inhibition of oxidative phosphorylation.

Initially, in studies on the effect AEC on enzyme activity, it was argued that the balance between ATP, ADP, and AMP was the key factor for cellular homeostasis. AEC was proposed as a means of comparing the physiological role of regulation of enzyme activities by adenine nucleotide ratios. The AEC expression assumes that the terminal phosphate groups of ADP and ATP are metabolically equivalent (Atkinson, 1968), which is true only if the adenylate kinase maintains a near-equilibrium state between adenylates (Pradet and Raymond, 1983). Nevertheless, in maize root tips acclimated to low-O_2, survival is not critically determined by nucleotide levels (Xia et al., 1995).

Modification of ATP-utilizing enzymes and glycolytic metabolism are two general means through which the relationships between nucleotide fluxes and nucleotide concentrations, or their ratios, could be altered (Xia et al., 1995). In asparagus tips, it seemed that all sub-ambient lower O_2 partial pressures affected AEC. However, the dramatic decrease in AEC as O_2 decreased below 2 kPa (Fig. 4.7), was accompanied by a similar trend for ATP:PFK and sugar-metabolizing enzyme activities (Silva et al., 1998b). These apparently coordinated alterations in enzymes and adenylate pool sizes may indicate that the balance between pathways of ATP regeneration and consumption has been altered. Furthermore, low O_2 may lead to cytoplasmic acidosis, which directly increases ATP hydrolysis, perhaps through activation of acid phosphatases (Roberts et al., 1984), resulting in reduced *in vivo* ATP:PFK activity (Raymond et al., 1985). Plasma membrane and tonoplast H^+-ATPases play an important role in intracellular pH regulation in plants (Xia and Roberts, 1994). Under acidosis, H^+-ATPases consume much more ATP to extrude protons that leak into the cell (Gout et al., 1992). Therefore, even in asparagus spear tips acclimated to O_2 partial pressures, below 2 kPa O_2, the activity of plasma membrane and tonoplast H^+-ATPases would be affected by extreme reduction in intracellular ATP levels, which may cause significant drop in cytoplasmic pH. Cytoplasmic pH may be affected by the effect of fermentation to lactate and H^+ transport. In plant tissues exposed to high CO_2, pH may also be reduced by carbonic acid dissociations to bicarbonate and hydrogen ion (Roberts et al., 1984). In maize seedling root tips, anoxia led to ATP breakdown without a concomitant increase in ATP synthesis via fermentation (Xia and Roberts, 1994). This agrees with the hypoxic and O_2-deficient

ATP profiles reported here for asparagus spear tips, where reduced O_2 availability progressively reduced ATP content (Fig. 4.4), AEC values (Fig. 4.7), and ATP/ADP ratios (Figs. 4.8 and 4.9), with paralleled increases in Pi (Fig. 4.3), ADP (Fig. 4.5) and AMP (Fig. 4.6). Hence, as also suggested for nonacclimated maize root tips (Xia et al., 1995), in asparagus tips below 2 kPa O_2, declining ATP may cause the plant material to respond by reducing hexokinase activity (Bouny and Saglio, 1996); Silva et al, 1998b), leading to reductions in glycolytic flux below 1 kPa O_2. In maize root tips acclimated with 3 kPa O_2 the ATP/ADP ratio dropped 30 to 50 % relative to air treatment (Xia and Roberts, 1994). A similar effect of O_2 was detected in asparagus. The decline in AEC and the ATP/ADP ratio may also indicate that other substrates, such as lipids, proteins and nucleic acids, were beginning to be utilized. The decrease in AEC here is consistent with data of Saglio et al. (1980) in hypoxic- treated maize root tips. Similar results were also reported by Brouquisse et al. (1991) in maize root tips. The latter authors suggested that, as the sugar supply is depleted and substrates other than sugars are utilized, enzymes from carbohydrate metabolism begin to be useless and become substrates for protein degradation.

In summary, the results here demonstrates that, in asparagus spear tips, as O_2 decreases below the critical level of 2 kPa and CO_2 partial pressure increases, Pi, ADP, and AMP contents increase, while PPi, ATP contents and AEC, ATP/ADP ratios decrease significantly. These alterations were all intensified at O_2 partial pressures below 2 kPa, which makes the transition to active fermentative metabolism. The data suggest 2 kPa is a critical O_2 threshold below which a marked modification in gene expression may occur.

These data, and the reduction of ATP:PFK activity (Silva et al., 1998b) and low AEC found below 1 kPa O_2, suggest an intracellular pH reduction. In addition, lower ATP content, AEC values, and ATP/ADP ratios at 20 kPa CO_2, at O_2 ranging from 2 to 16 kPa, indicate that high CO_2 may cause impairment of oxidative phosphorylation and limit cell survival, even at 16 kPa O_2. On the other hand, the marked drop in PPi and ATP contents associated with reductions in AEC, and ATP/ADP ratio, for all CO_2 treatments, at O_2 partial pressures below 2 kPa, indicates the critical role of O_2 in asparagus storage. This also suggests unbalanced cell metabolism, and a low protein turnover rate, which likely lead to cell death. This suggestion is further supported by the appearance of water soaked areas in asparagus spear tips under these low O_2 tensions (Silva et al., 1998a). Collectively, this data indicate different roles for CO_2 and O_2 along the range of steady state O_2 partial pressures studied. CO_2 effect seemed to predominate mostly from 2 to 16 kPa O_2, while O_2 effect seemed to predominate over that of CO_2 effect below 2 kPa O_2.

Finally, the roles that transcriptional and translational events play in determining the changes in pools of metabolites, metabolic energy, and enzyme activities, have yet to be established. Some of these modifications, such as reduction in ATP/ADP ratio with concomitant release of phosphate, may indicate the presence of an O_2 "sensor" (Hochachka et al., 1996) or may play a role in signal transduction. Repression of metabolic activity, protein synthesis and degradation are adaptative responses to hypoxia. Nevertheless, the limited number of enzymes directly involved (Ricard et al., 1991) makes anaerobic induction an attractive system in which to study the molecular mechanism of cellular adaptation to limiting energy availability.

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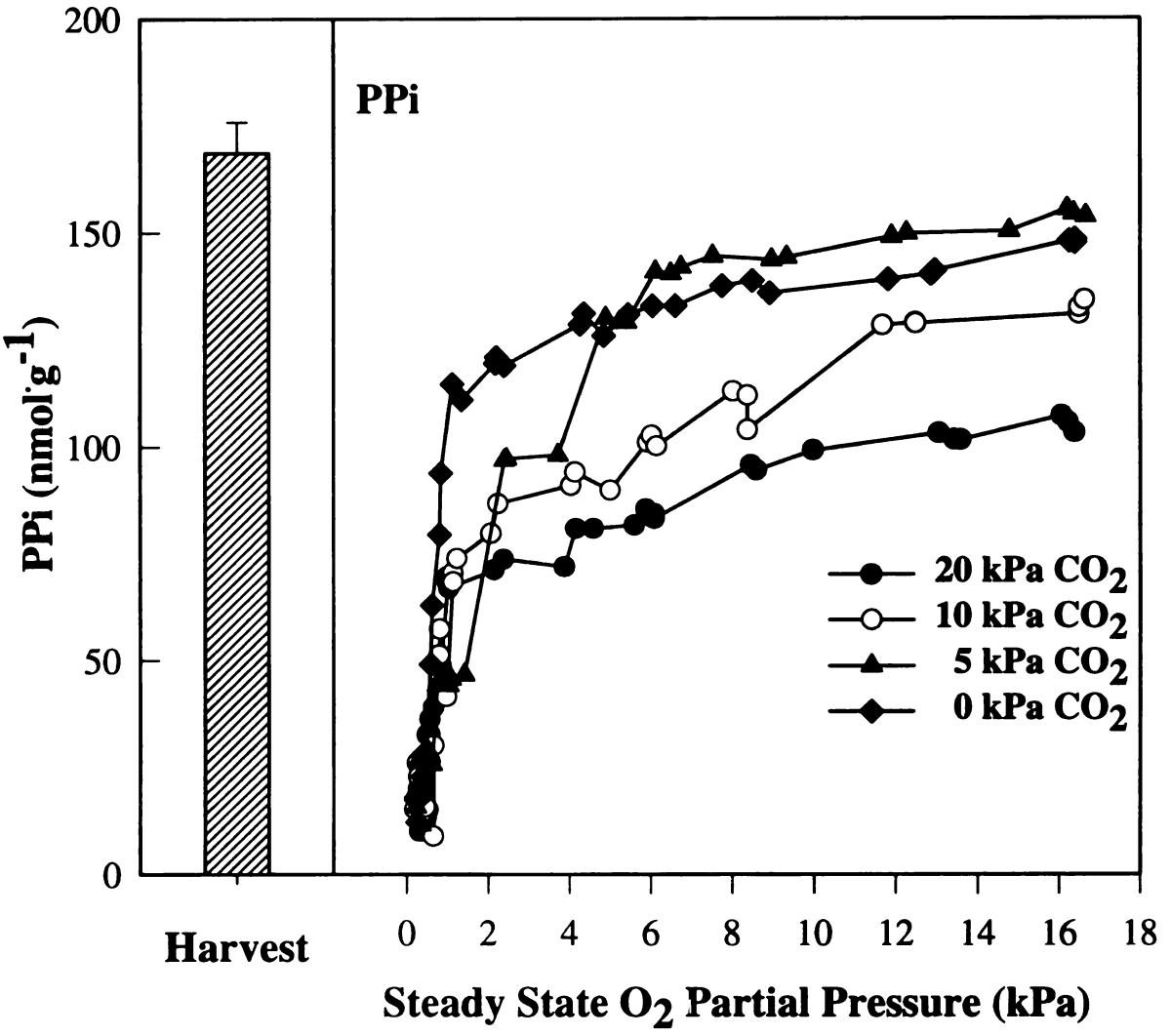

Figure 4.1. The effect of storage O_2 and CO_2 on the content of PPi (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

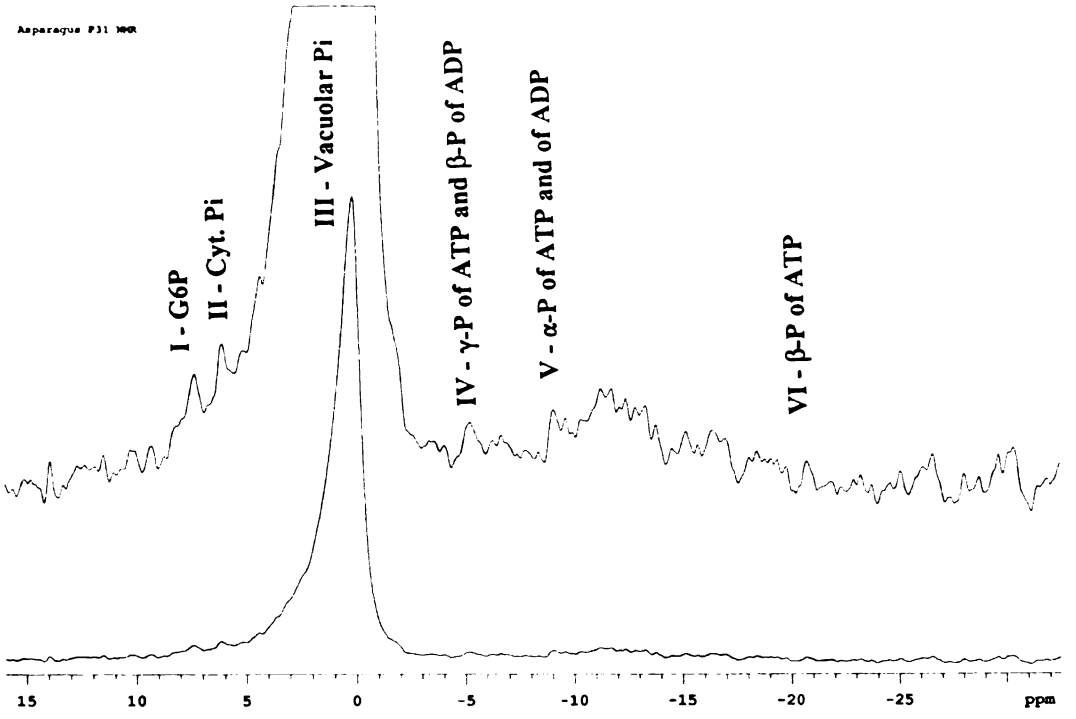

Figure 4.2. Pi status measured by ^{31}P-NMR, relative to harvest in 45 mm long asparagus spear tips under 1 kPa steady state O_2 and 20 kPa CO_2 held at 1 °C. Peak assignments are as follows: I, glucose-6-phosphate (G6P); II, cytoplasmic inorganic phosphate (Pi); III, vacuolar phosphate; IV, γ-P of ATP and β-P of ADP; V, α-P of ATP and of ADP; VI, β-P of ATP. Insert shows an amplified scale spectrum to improve peak discrimination.

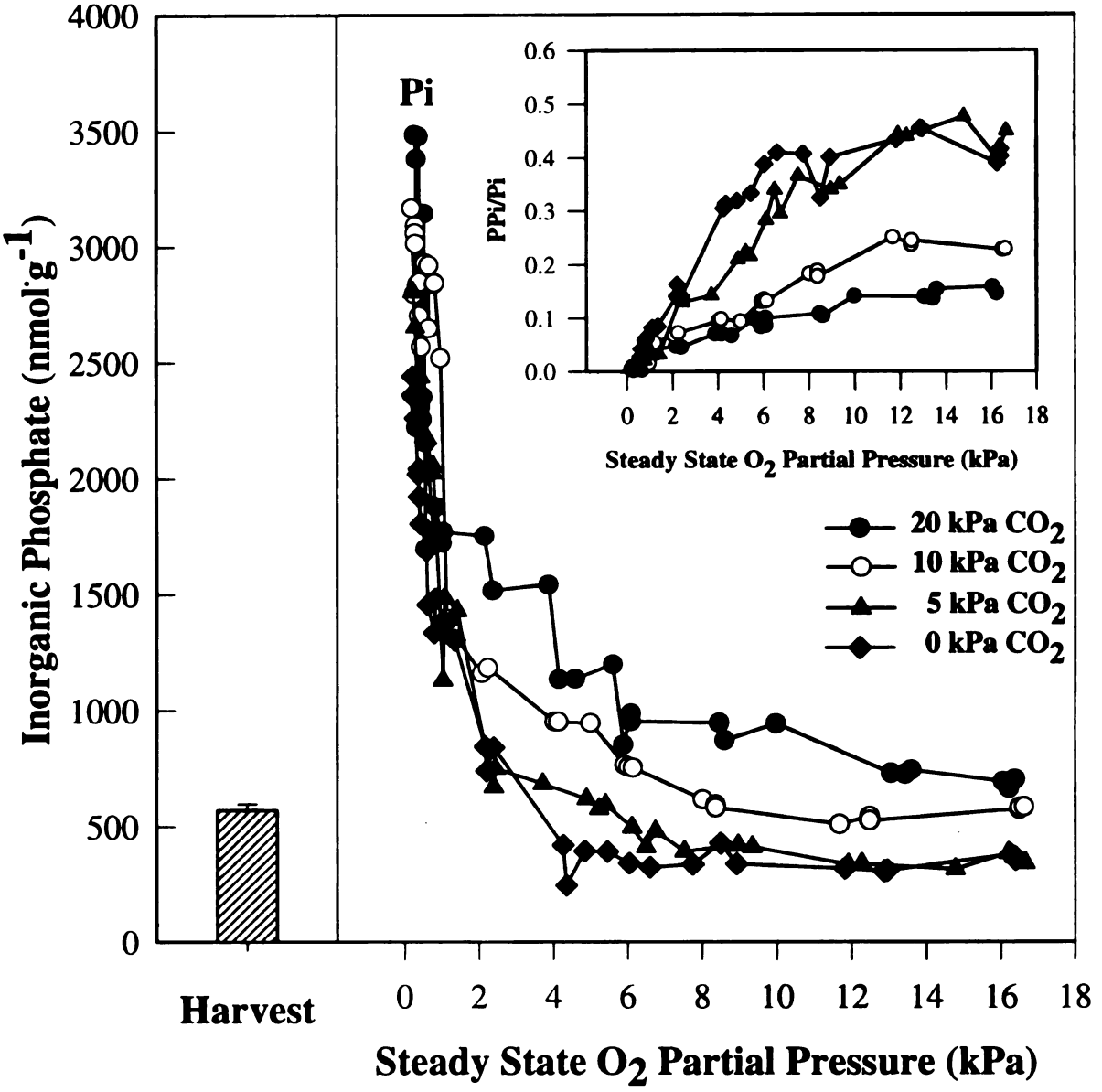

Figure 4.3. The effect of storage O_2 and CO_2 on the content of Pi (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days. Insert shows the effect of O_2 partial pressures on the PPi/Pi ratio.

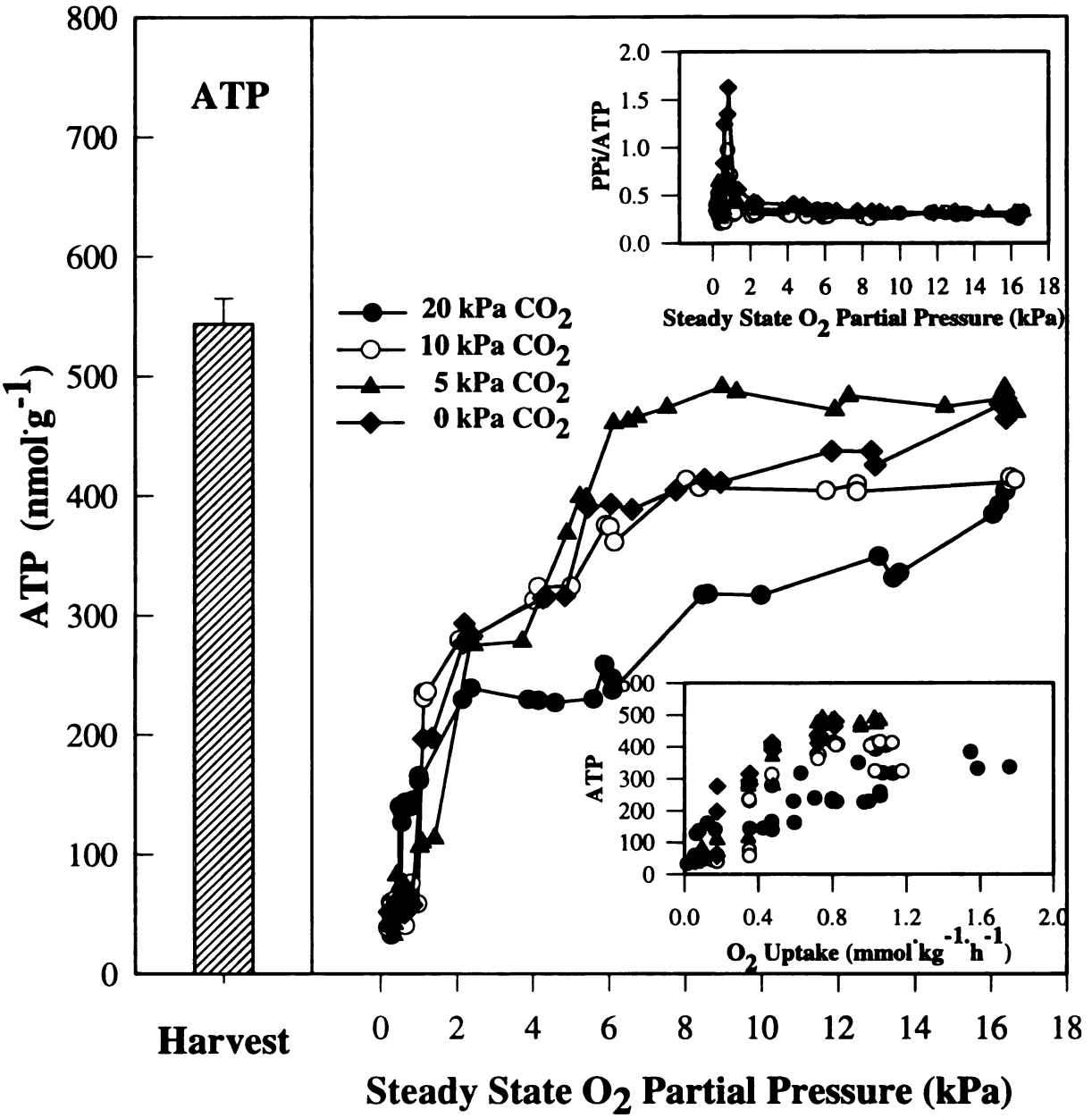

Figure 4.4. The effect of storage O_2 and CO_2 on the content of ATP (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days. Inserts show: A) the effect of O_2 partial pressures on the PPi/ATP ratio and B) the effect of O_2 uptake on the content of ATP.

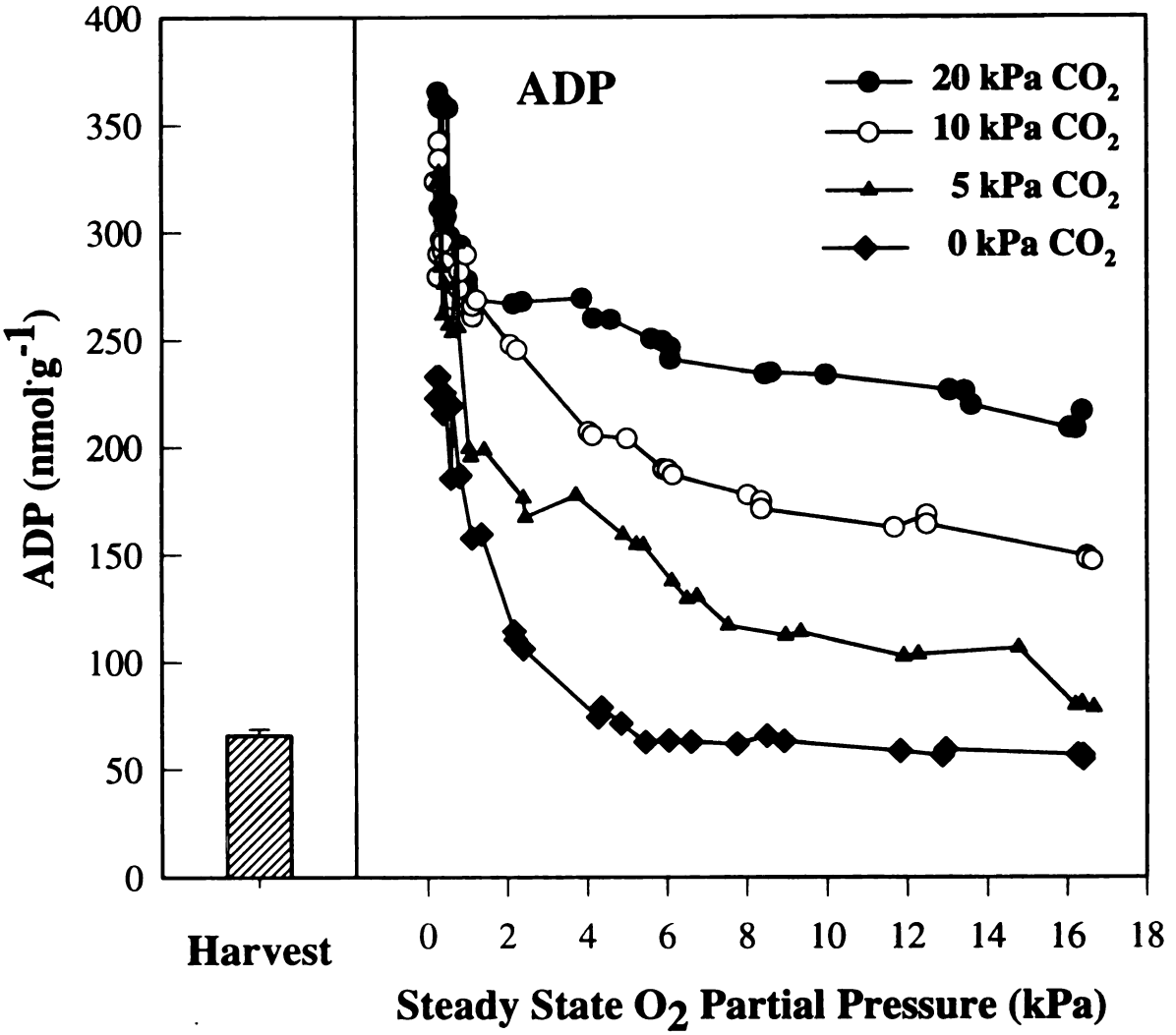

Figure 4.5. The effect of storage O_2 and CO_2 on the content of ADP (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

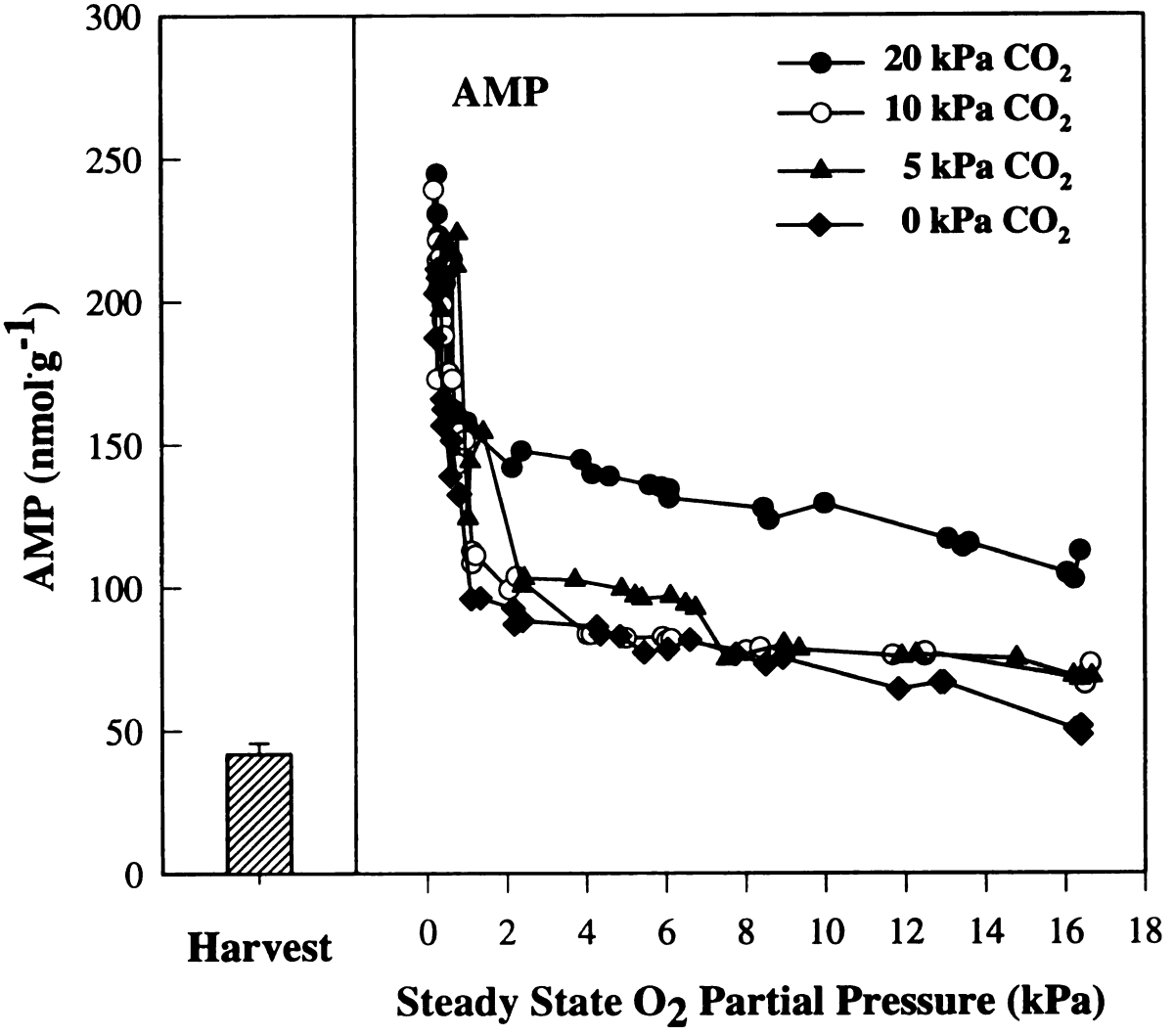

Figure 4.6. The effect of storage O_2 and CO_2 on the content of AMP (fresh mass basis), relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

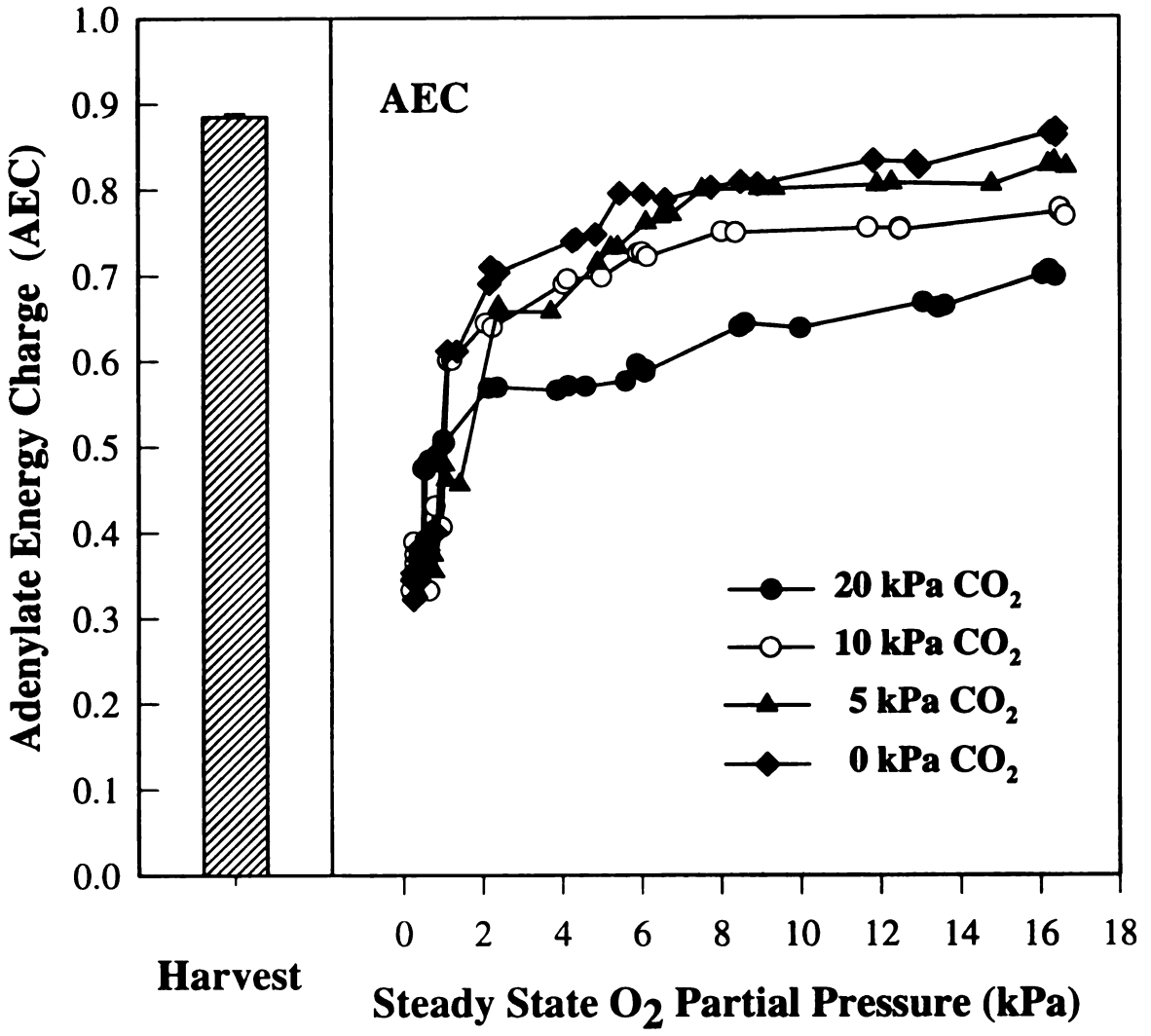

Figure 4.7. The effect of storage O_2 and CO_2 on the AEC, relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

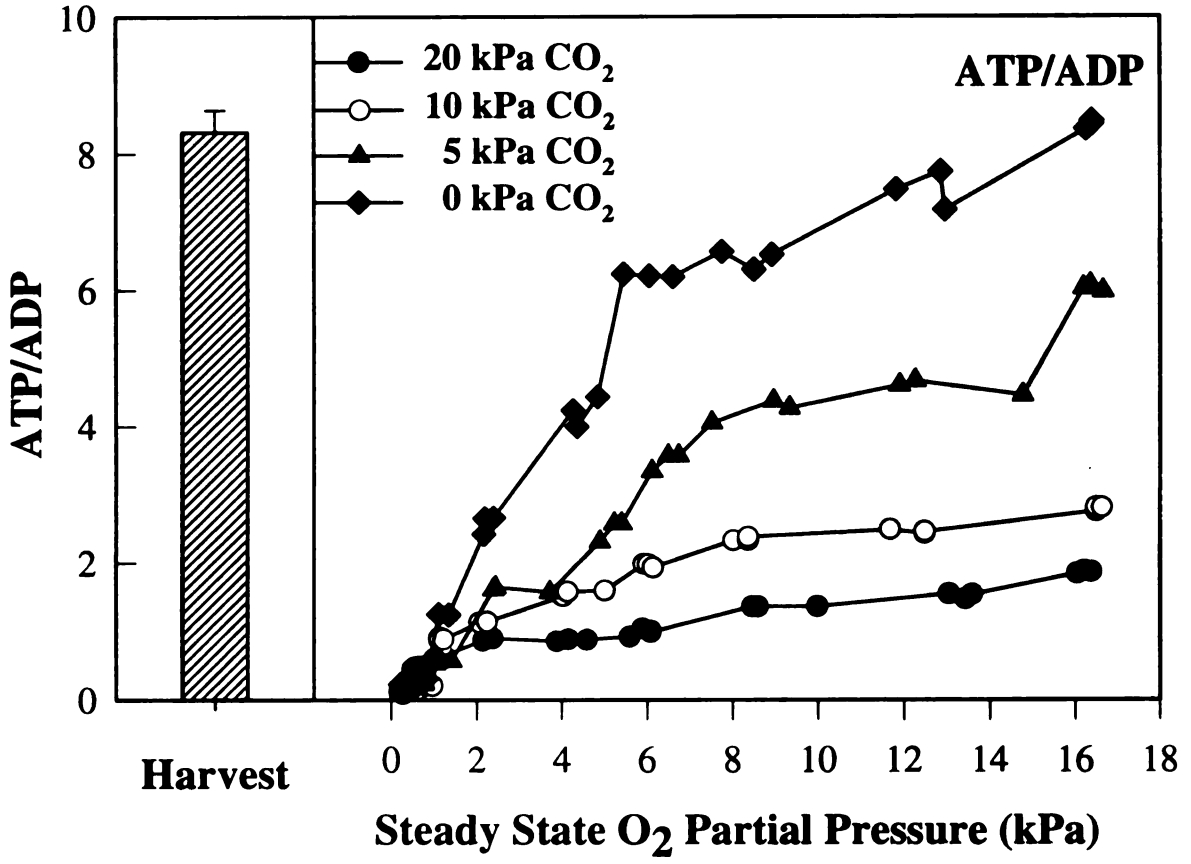

Figure 4.8. The effect of storage O_2 and CO_2 on the ATP/ADP ratio, relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

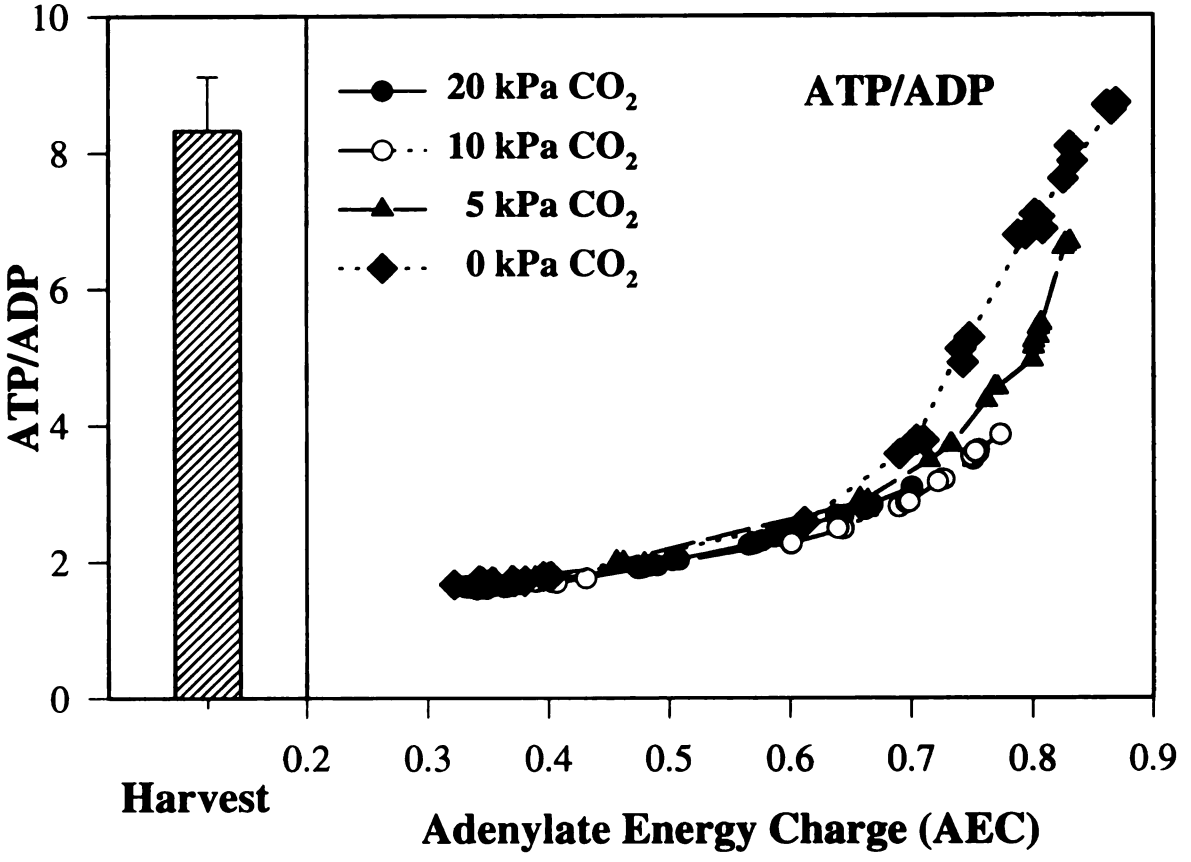

Figure 4.9. The effect of AEC on the ATP/ADP ratio, relative to harvest in the most acropetal 45 mm of the tip of asparagus spear held at 1 °C for 6 days.

CHAPTER 5

EFFECTS OF 6-BENZYLAMINOPURINE ON SUGAR PROFILE AND SENESCENCE OF ASPARAGUS (*Asparagus officinalis* L.) SPEARS STORED AT 1 °C

INTRODUCTION

Asparagus spears senesce rapidly postharvest (Lipton, 1990). Postharvest physiological changes include a decline in respiration rate, a drop in soluble carbohydrates, mainly sucrose, and losses in chlorophyll, protein and amino acids. Although senescence is a genetically controlled and programed process (Thomas and Stoddart, 1980), it is subject to modifications through external influences (Crafts-Brandner, 1984). Exogenous application of cytokinins to plant tissues results in a variety of responses which may include a delay in senescence, increased chlorophyll production, a stimulation of chloroplast development, maintenance of chloroplast activity, a reduced chlorophyll degradation, the promotion of protein and nucleic acid synthesis, and the mobilization of nutrients into the cytokinin-treated area (Clarke et al., 1994; Müller and Leopold, 1966; Wingler et al., 1998). The extension of the shelflife for cytokinin-treated plant tissues has been attributed to lower respiration rates compared to control (Rushing, 1990); however, changes in gene expression may also explain those responses (Chen and Leisner, 1985; Van der Werf and Nagel, 1996).

The senescence-delaying effect of exogenous cytokinin has been derived from classical experiments which demonstrate the correlative evidence linking endogenous levels of cytokinins to the progress of leaf senescence in corn leaves (Müller and Leopold, 1966). However the most convincing evidence for role of endogenous cytokinins in

senescence comes from transformation experiments in which a significant delay in leaf senescence was observed in transformed plants containing an active bacterial cytokinin synthase gene and elevated endogenous cytokinin levels (Clarke et al., 1994). Van der Werf and Nagel (1996) hypothesized that allocation of carbon is mediated by sucrose and cytokinins. It has also been suggested by Farrar (1996) that sucrose not only plays a role in allocation of assimilates between sources and sinks, but also functions as an indicator of the assimilate status of both source and sink. Sugar concentration may also exert an influence in the expression of some genes to permit matching the availability of sugars from the source and the demand of carbon of the sink (Van der Werf and Nagel, 1996). Since the tip of spear functions as the sink for harvested asparagus (King et al., 1990), and sugars from the lower portions of the spear supply the carbohydrate demand of metabolic processes of the tip (Saltveit and Kasmire, 1985), the utilization of carbon by this crop may be responsive to status of cytokinin and sucrose in the spear tip.

A reduced rate of cell division in leaves leads to a reduced unloading of sucrose from the phloem into the expanding cells. Consequently, as sucrose concentration in the phloem nearby the expanding cells increases, it leads to an increase of the turgor pressure in the cell division zone, at the phloem adjacent cells, resulting in expansion growth. Supporting this idea, Downes et al., (1969), described the effects of temperature in the rate of growth of asparagus spears as related to the gain in weight and anatomical changes. Their results also suggested that the tip may exercise some influence in the rate of elongation of the stem.

The objective of this research was to evaluate the influence of tip presence and

exogenous 6-BAP application on carbohydrate metabolism and visual quality on stored asparagus spears. CO_2 production, chlorophyll fluorescence, chlorophyll content, soluble sugars, and visual quality were measured during 31 days of storage at 1 °C.

MATERIAL AND METHODS

Plant material and treatments. Asparagus (*Asparagus officinalis* L. cv. Jersey Giant) spears were harvested in May 1996 no later than 9:00 a.m. from three year old plants at the Michigan State University Horticultural Teaching Research Center. Spears were surface-sterilized in the field by dipping for 1 min in 150 ppm NaOCl followed by a rinse with tap water. Sterilized spears were placed in contact with absorbent paper to remove excess moisture. Two treatments were imposed: tip removal and exogenously applied cytokinin. In addition to control spears, there were three treatment combinations (6BAP, tipped, 6BAP + tipped). Initially, the length of all spears was adjusted to 180 mm. Tipped spears had a 45 mm length of the apex was removed using a sharp, stainless steel knife. Cytokinin was applied in the field immediately following sterilization, using 4.42 x 10^{-4} M (100 ppm) of 6-BAP (Sigma Chemical Co., St. Louis, MO, USA) predissolved in minimal dimethylsulphoxide prior to dilution in water with 0.02% Tween 20 (Nutritional Biochemical Corporation, Cleveland. OH, USA). 6-BAP was applied by placing cheese cloth soaked in 6-BAP in contact with the spear for 30 seconds. Excess solution was removed by absorbent paper. Treated and untreated spears were placed in an ice-cold chest and transported to the laboratory. Spears were held in the dark at 1 °C. For each assay date spears were placed in liquid nitrogen at harvest and stored at -80 °C for later

measurement.

Spears of each treatment combination were divided into two lots. One lot was used to monitor the respiratory rate. The second lot was used as a source of spears for other assays including sugar, chlorophyll fluorescence, chlorophyll content, length, and visual quality. These assays were performed for spears stored 0, 3, 7, 12, 17, 26, and 31 days.

Respiration rate measurement. Three replicates consisting of ten spears were enclosed in 1.980 L mason jars ventilated with air at a flow rate of 30 $mL \cdot min^{-1}$. The CO_2 concentration of the exit gas stream was determined on a 100 µL gas sample taken with a 0.5 mL insulin-type syringe. CO_2 analysis was done by an infrared gas analyzer (Model ADC 225-MK3, The Analytical Development Co. Ltd., Crowborough, Sussex, England) with N_2 as the carrier gas at a flow rate of approximately 100 mL·min (Beaudry, 1993). Respiration rates were measured approximately 30 min after harvest (time 0), and on a daily basis for 31 days. Extra jars, similar to those described above, were prepared for evaluation of the other parameters following described, and at similar time course.

Chlorophyll fluorescence determinations. Chlorophyll fluorescence was measured at harvest and at regular time intervals from base to tip, using a pulse-modulated fluorometer (Model OS-500, Opti-Sciences, USA). Measurements were taken from the center of 45 mm long four segment spears. Three readings in equidistant locations of each section were taken and averaged. Six spears were used for make up the average for each replication. Spears were acclimated in the dark for 1 hour room temperature and measurements were made in a darkened laboratory. Fluorescence was measured using a

photodiode in the wavelength range of 710-760 nm. For each measurement run, minimal fluorescence (Fo) was monitored for 0.2 sec at a rate of 100 readings per second (20 sample points). The excitation (modulated) light (660 nm, filter for > 700nm light) intensity for the Fo measurement was approximately 0.15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (OS-500 setting = 60) which was sufficient to get an accurate measurement of Fo (Van Kooten and Snel, 1990). The addition of approximately 3 mW (OS-500 setting = 50) of continuous far red light (735 nm) did not influence fluorescence, therefore it was assumed that the redox components of the electron transport chain were fully oxidized. After Fo was determined, the sampling rate was then increased to $1000 \cdot s^{-1}$ and a saturation light (660 nm) pulse was supplied via the light guide by a halogen lamp. The light intensity at the peel during the pulse was estimated to be 2,400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) based on an OS-500 setting of 60; a setting of 255 yields approximately 10,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. The pulse duration was 0.8 s and a cut-off filter, blocking light above 700 nm, was used to prevent saturation of the photodiode. During the pulse, the maximum level of modulated chlorophyll was designated as Fm. The efficiency of photosystem II (Fv/Fm) was calculated as (Fm-Fo)/Fm (Van Kooten and Snel, 1990).

Chlorophyll determination. Chlorophyll content was determined at the same time intervals and in the same four 45 mm long spear sections used for chlorophyll fluorescence. For this determination, approximately 500 mg of the fresh epidermis tissues were removed and extracted in 500 mL of N,N-dimethylformamide (Sigma Chemical Co., St. Louis, MO, USA) for 24 h at 4 °C according to Moran and Porath (1980). Chlorophyll measurement were made using a spectrophotometer (Model U-3110, Hitachi

Ltd, Tokyo, Japan) at 603, 647, and 664 nm. The estimation for chlorophyll a, b, and total were calculated from formulae described by Moran (1982).

Soluble sugars determination. Soluble sugars were extracted from four 45 mm long segments which were previously stored at - 80 °C. The segments were the tip, and proceeding basipetally, 2-4 were successive 45 mm length with segment 4 being the spear base. Sugars were extracted from grounded spear segments in 3.5 mL of 80% ethanol for 15 minutes at room temperature. The mixture was centrifuged (Model RG-5C, Sorval Instruments, Dupont Co., Newtown, CT, USA) at 2,000 *g* for 5 minutes and the supernatant decanted into a capped 50 mL centrifuge tube. This procedure was repeated two times and the extract obtained was washed out with 5 mL of water. To remove chlorophyll, 5 mL of chloroform was added to the combined 15.5 mL ethanol /water mixture. The tubes were capped, shaken vigorously and then centrifuged at 1,000 *g* for 3 minutes. The upper, clear aqueous phase containing the soluble sugars was transferred to a test tube and evaporated to dryness using a speedvac equipped with a refrigerated condensation trap (at -103 °C) (SC200 Speedvac, Savant Instruments Inc., Farmingdale, NY).

The dried soluble carbohydrates were solubilized overnight with agitation in 1 mL of dry pyridine containing 30 $mg.mL^{-1}$ hydroxylamine hydrochloride, heated at 70 to 80 °C for 1 h, and derivatized. Carbohydrates were assayed using a gas chromatograph (HP Model 5890 series II, Hewlett-Packard Co. Palo Alto, CA) fitted with a FID. The column (DB-1701 capillary column, J &W Scientific, Folsom, CA) had a 0.25-µm thick coating, was 30m in length x 250 µm i.d.

Sensory quality. A continuos visual rating scale was constructed to evaluate the visual quality. Whole spears were rated 12 h after being removed from the jars at room temperature. Each treatment replication was rated independently by the subjective evaluation of four observers using the following scale adapted from Silva et al. (1998):

9= fresh like;

7= slight chlorophyll loss in tips and scales, slight flaccidity and/or very slight wrinkle on stems, absence of strange flavors, no feathering due to irregular growth;

5= moderate chlorophyll loss and browning in tips and/or stem bracts browning, more pronounced wrinkle, loss of pink blush at the base, slight ethanol odor, slight feathering due to irregular growth;

3= extensive chlorophyll loss, browning, and pronounced flaccidity of tips and stems bracts (feathering), wrinkle and wilting of stems, moderate fermentative odor moderate feathering due to irregular growth;

1= extensive loss of chlorophyll, browning and flaccidity of tip and stems bracts, complete loss of pink blush at the base, extensive wrinkle and wilting stems and base, brown spot in stems when exposed to air, strong fermentative odor, extensive feathering due to irregular growth.

A rating of 6 (threshold quality) was considered unacceptable for sale although still edible.

RESULTS AND DISCUSSION

Carbon dioxide production. Relative to spears with tip, at 0 d, CO_2 production was 35% higher in spears without tips. CO_2 did not differ for spears with and without tips after 3 d. 6-BAP applications, however, resulted in lower CO_2 production in spears, with and without tips (Fig. 5.1). Wounding of tissues caused by cutting and injuries usually results in increased CO_2 production (Salveit and Kasmire, 1985), an effect observed here for spears without tips. However, even though CO_2 production in spears without tips was markedly higher than those with tips at the beginning of the storage, the respiratory rates were lower for cytokinin-treated spears, indicating that cytokinin application reduced respiration. CO_2 production is understood as a physiological indicator of the metabolic activity of a crop (Kays, 1991) and its decline is usually associated with a general decline of the metabolic activity of the whole system. Thus, the results presented here suggest that exogenous cytokinin application has the potential to reduce the metabolic rate of asparagus spears postharvest. On the other hand, CO_2 production did not differ among treatments after 10 days and throughout the remainder storage period at 1 °C. 6-BAP has a similar effect on CO_2 production in broccoli stored at 16 °C (Rushing, 1990).

Soluble sugars. In spears with intact tips, fructose content in sections 2, 3, and 4 remained lower in cytokinin-treated spears (Fig. 5.2). In the tips, however, fructose content was higher for cytokinin-treated spears for the first 5d of storage and did not differ therafter. In spears without tips, fructose did not differ in section 2 for cytokinin-treated and untreated spears. However, in cytokinin-treated tipped spears, fructose content was slightly higher in section 3 during the later portion of the storage and in

section 4, markedly higher for the first 12 d of storage (Fig. 5.3). For spears with and without tips, 6BAP did not affect the glucose profile, except in section 4 of intact spears which experienced a reduction in glucose in response to 6-BAP treatment (Figs. 5.4 and 5.5). Sucrose content was markedly affected by cytokinin treatment, but only in the location to which it was applied. Its content was higher in the tips of cytokinin-treated spears (Fig. 5.6). This response differs from that of Irving and Joyce (1995) in broccoli stored at 23 °C. These results suggest that, in asparagus spears, cytokinin applications may either cause mobilization of sucrose from lower sections to the tip of spears or reduce the activities of sucrose-metabolizing enzymes, minimizing degradation of this sugar. It has been suggested that cytokinin, in addition to delaying senescence, could block some of the responses to sugar levels (Jang et al., 1997). In addition, carbon allocation to shoots and roots may be mediated by cytokinin and sucrose (Van der Werf and Nagel, 1996). If cytokinin causes a change in sucrose allocation, it would be possible, by evaluating the segmental spear sugar profile, to observe a changed import of sugars into the cytokinin-responding sink tips. However, since the hexose content in the tip (Figs. 5.3 and 5.5) did not change much upon cytokinin treatment, it is difficult to determine if the delay in the sucrose decline in the tip was caused by its import from lower sections or by gluconeogenesis from other substrates. When submitted to carbohydrate starvation, plant cells may adapt, and substitute protein and lipid metabolism through autophagic processes (James et al., 1993; Moriyasu and Ohsumi, 1996; Saglio and Pradet, 1980). On the other hand, in spears without tips, section 2 of the cytokinin-treatments tended to accumulate more sucrose than untreated in the first 20 d of storage (Fig. 5.6); however, the behavior

of the other sections was not much different from non-treated spears. Sucrose utilization following storage appeared to be reduced in spears without tips, indicating that the tip may have a role in the postharvest senescence of spears.

Sucrose decline in asparagus tips postharvest has been suggested to trigger senescence for the whole spear (King et al., 1990). The ability of sink organs to competitively attract assimilates is termed sink strength (Ho, 1988). Sink strength is determined by the metabolic activity in the sink tissue, and by a number of enzyme activities playing a key role in regulation in sink activity (Nielsen and Ulvskov, 1992) and sink-strength regulation (Wingler et al., 1998). The asparagus spear tip, being comprised of highly meristematic tissue (Robb, 1984), acts as a strong sink, and is characterized as having highly active metabolism (King et al., 1990). The change in the pattern of sucrose utilization in cytokinin-treated spears suggests that in some way cytokinin caused a blockage in the sensor that triggers sucrose breakdown and utilization. In transgenic tobacco, for example, it was reported that sugars, cytokinin, and light interact during senescence by influencing the decline in proteins involved in photosynthetic metabolism (Wingler et al, 1998). The results in harvested asparagus show that the decline in sucrose content was, however, closely associated with a decline in respiration rate and with a decline in chlorophyll content and changes in chlorophyll fluorescence, indicating that a series of processes may be affected by the sucrose status and sugar utilization in asparagus spears.

Changes in chlorophyll content. Cytokinins are known to affect photosynthesis and to stimulate chloroplast biogenesis and chlorophyll biosynthesis, promoting the

synthesis of RuBPCase, the differentiation of chloroplasts, and the expression of genes encoding the small subunit of RuBPCase and the light harvesting chlorophyll *a*/*b*-binding proteins (Genkov et al., 1997; Nielsen and Ulvskov, 1992; Wingler et al., 1998), resulting in slower rates of chlorophyll degradation. Our results also confirm that applied 6-BAP delayed the degreening of asparagus spears stored at 1 °C (Figs. 5.8 and 5.9). 6-BAP application also promoted chlorophyll retention in de-rooted oat seedlings (Badenoch-Jones, et al., 1996) and in meristematic floral tissues of broccoli (Irving and Joyce, 1995; Clarke et al., 1994). Chlorophyll *a* content was the major contributor to total chlorophyll content, and was four-fold higher than chlorophyll *b* (data not shown). When treated with 6-BAP, the spear tips appeared to accumulate more chlorophyll *a* than other sections. Therefore, application of 6-BAP in asparagus spears tips not only delayed chlorophyll degradation but also changed the pattern of chlorophyll distribution and metabolization by the spear.

Changes in chlorophyl fluorescence. Chlorophyll fluorescence has been used as an indicator of postharvest chilling injury in banana and mango (Smillie et al., 1994), tomato (Jung and Steffen, 1997), and green bell peppers (Lurie et al., 1994), and of scald in apples (DeEll et al, 1996). In spears with tips, Fo, the basal fluorescence of dark-adapted spears, started to increase after 15 d storage in untreated spear tips compared to more than 25 days in cytokinin-treated spears. Fo was not affected by 6-BAP for spears without tips (Fig. 5.10). The removal of the tip did seem to affect Fo values for sections 2, 3, and 4 for cytokinin-treated and -untreated spears. In cytokinin-treated spears, Fm, the maximum fluorescence of dark-adapted spears, did not decrease after 15 d storage, but

started to decreased after the same period in untreated spears. However, the decline was much more pronounced is spears without tips (Fig. 5.11). Reduction in the ratio Fv/Fm is manifested as a reduction in the quantum yield of PSII in a decrease in variable chlorophyll *a* fluorescence (Demmig and Björkman, 1987). In spears with tips, Fv/Fm, an indicator of the photosynthetic capacity of PSII, was significantly higher in cytokinin-treated than in untreated spears within 10 days of storage. This ratio was also higher for the section 2 of 6-BAP-treated spears which had the tips removed (Fig. 5.12). Reductions in Fm and Fv/Fm indicated severe damage in PSII (Franklin et al., 1992). The observed reduction in Fv/Fm and the increase in Fo during storage for untreated spears, indicated that there was damage to the PSII centers that was not readily reversible (Jung and Steffen, 1997). It is unlikely that cellular repair mechanisms could match the rate of damage to the PSII and hence photosynthetic capacity was impaired (Layne and Flore, 1993). In addition, the decreased efficiency of PSII photochemistry during storage in untreated spears may reflect the inhibition of PSII function and an increase in thermal radiationless energy dissipation (Jung and Steffen, 1997), which appeared to be lessened in cytokinin-treated spears.

Spear growth. Cytokinin promotes the growth of excised cotyledons of various plants, particularly in darkness (Ulvskov et al., 1992; Chen and Leisner, 1985). The most common responses of the cotyledons to hormonal stimulation is cell expansion (Huff and Ross, 1975). The results here indicate that cellular expansion also occurred in asparagus spears stored at 1 °C, with cytokinin stimulating growth of approximately 8% (Fig.5.13). It has been suggested that cytokinins may affect growth by increasing tissue ability to take

up, retain or metabolize available assimilates, which would result in changes in sink strength. This additional growth could be result from an increased biosynthesis of cell components required for the division and expansion of the cell (Nielsen and Ulvskov, 1992) and promotion of anatomical changes (Downes et al., 1964). Alternatively, increased solute import could be the basis of decreasing water potential. This would lead to increased turgor pressure, which potentially could be the driving force for cell enlargement (Ulvskov et al., 1992). Although the application of cytokinin resulted in irregular growth of the spears, this did not much influence their qualitative acceptance, as noted by its visual quality (Fig. 5.14). In addition, the data suggest that the tip has a marked influence in promoting cell enlargement, since its removal inhibited growth.

Changes in sensorial quality. Application of 6-BAP to the treated surface of the spear delayed senescence as ascertained by the maintenance of the visual appearance. 6-BAP-treated spears with tips dropped below the acceptable visual quality threshold 10 days earlier than untreated spears (Fig 5.14). Exogenous cytokinin also delayed senescence, as measured by appearance, in de-rooted oat and wheat seedlings, and was suggested to have a role in regulating senescence in intact seedlings (Badenoch-Jones et al., 1996). However, they reported that such a cytokinin effect is highly species-specific. The results here indicate that the tip is the segment most affected by senescence, probably due to its being the most metabolically active tissue. In addition, spears without tips had much lower acceptance, suggesting that good visual quality for asparagus spears requires the presence of the tip.

Collectively, cytokinin application to the tips minimized sucrose depletion and

other measures of senescence in asparagus. The results showed that the direct application of 6-BAP delayed chlorophyll loss, decreased respiration rate, delayed changes in chlorophyll fluorescence, improved the general appearance, and minimized the loss of sucrose in spears with tips. The carbohydrate data indicates that cytokinins affect carbon transport, uptake and metabolism. Further research, however, is required to determine how cytokinin is able to modify the pattern of sucrose utilization by asparagus spears during storage at 1 °C.

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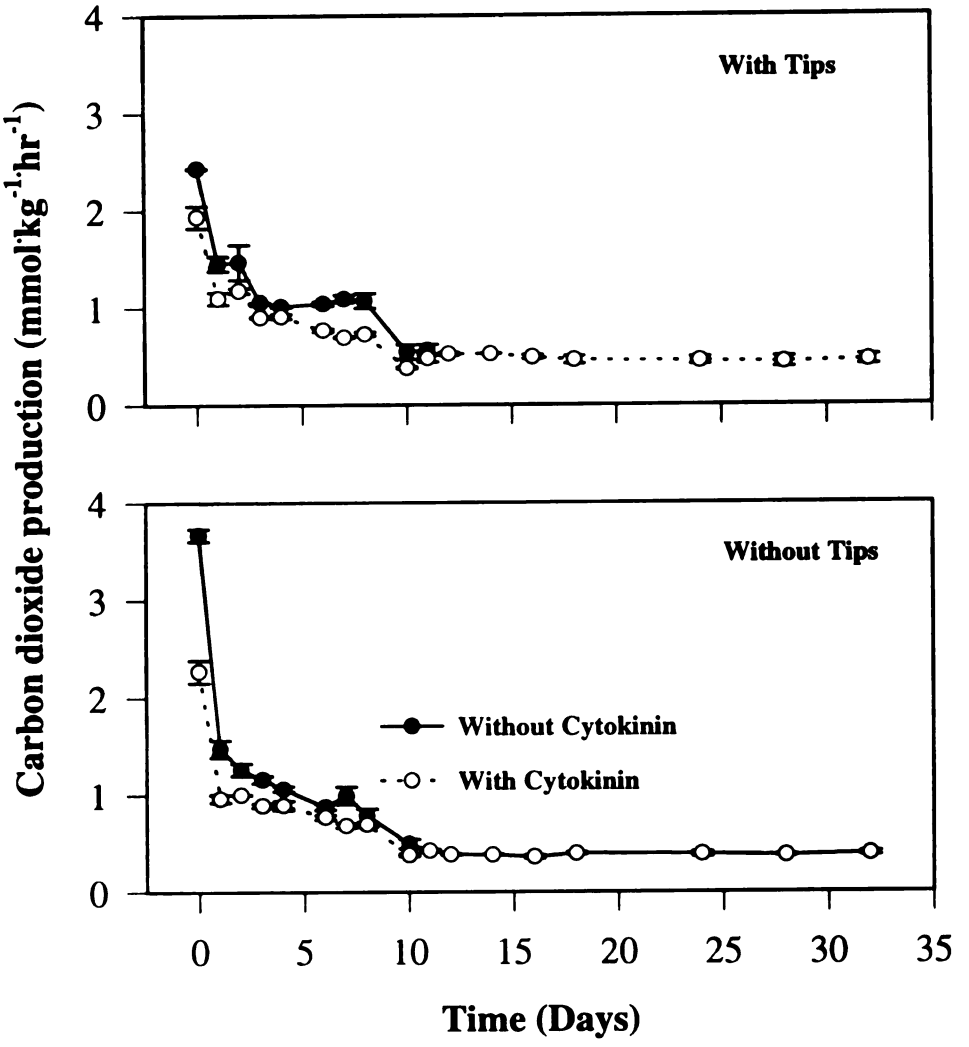

Figure 5.1. Changes in carbon dioxide production in asparagus spears with and without 6-BAP, with and without 45 mm long tip segments during storage at 1 °C. Bar = ± SE(n=3).

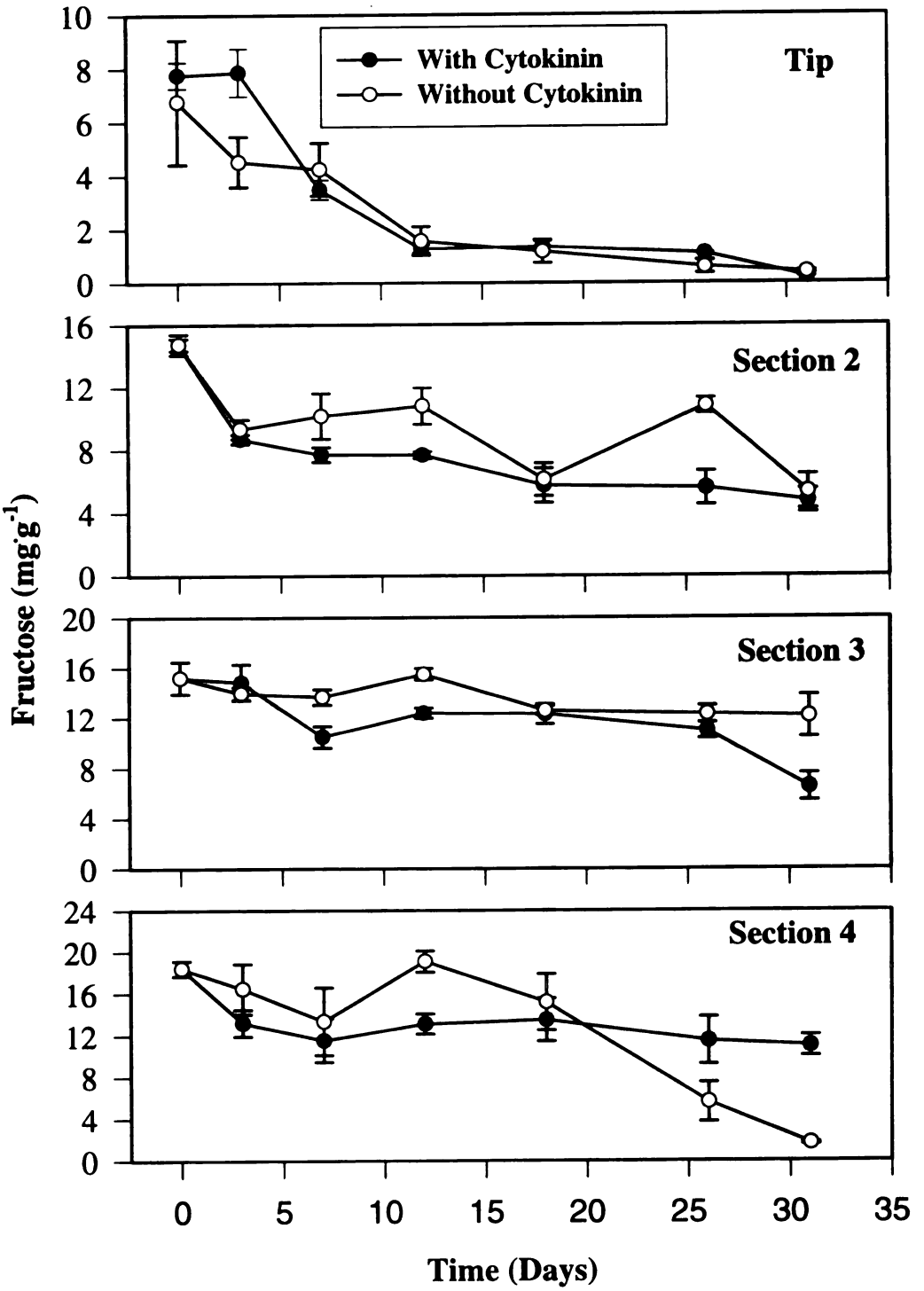

Figure 5.2. Changes in fructose content (fresh mass basis) in asparagus spears with tips, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

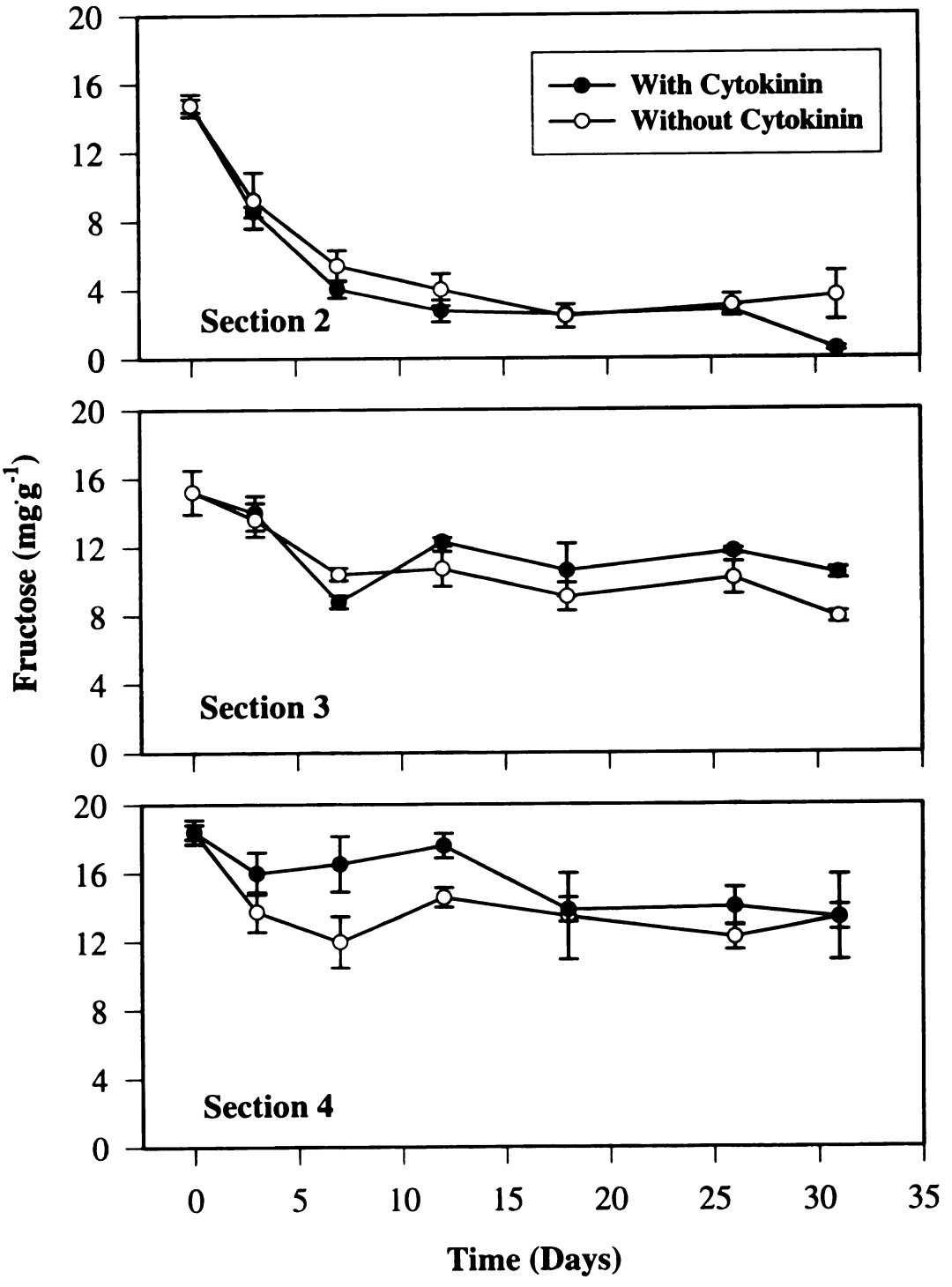

Figure 5.3. Changes in fructose content (fresh mass basis) in asparagus spears without 45 mm long tip segments, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

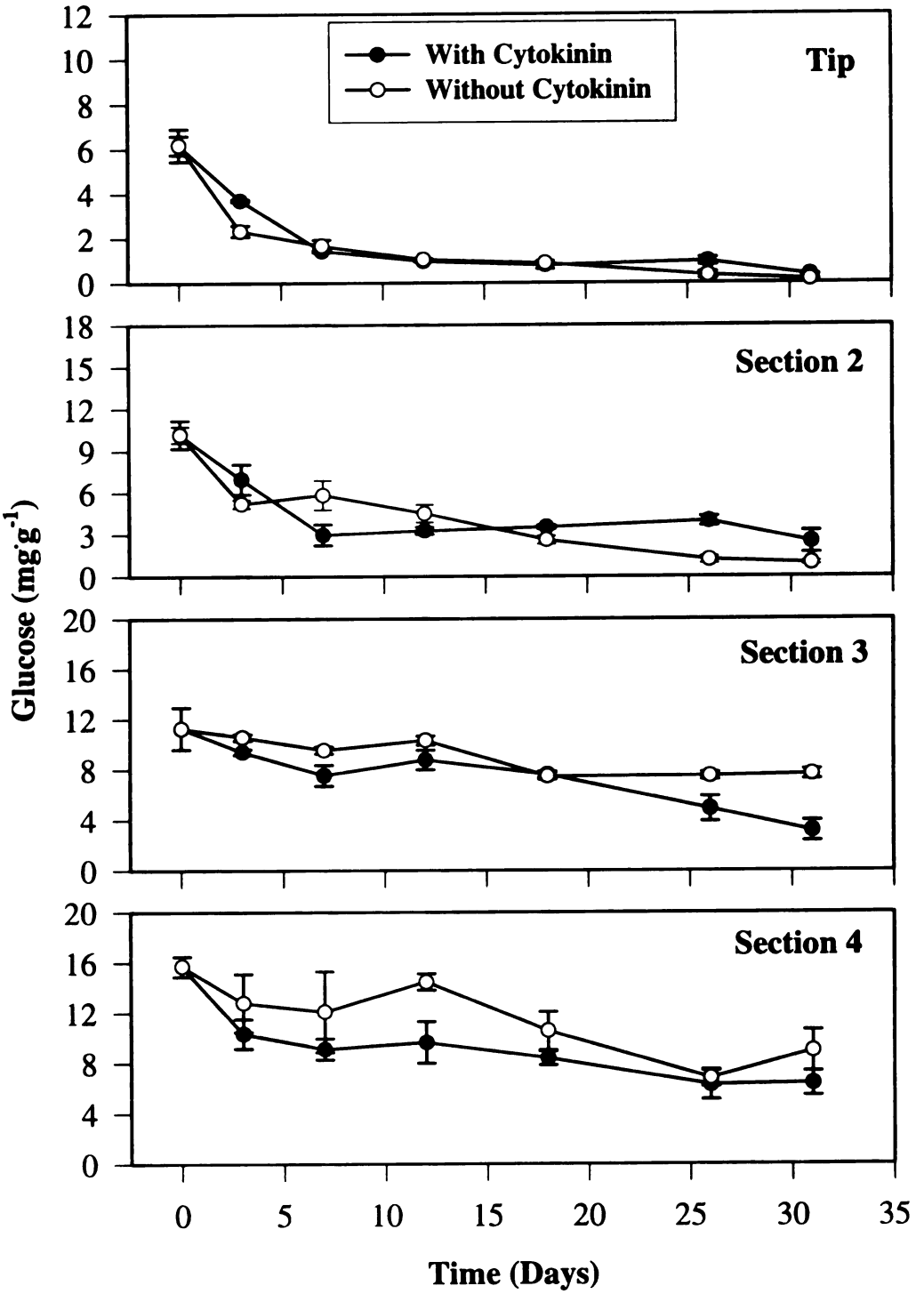

Figure 5.4. Changes in glucose content (fresh mass basis) in asparagus spears with tips, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

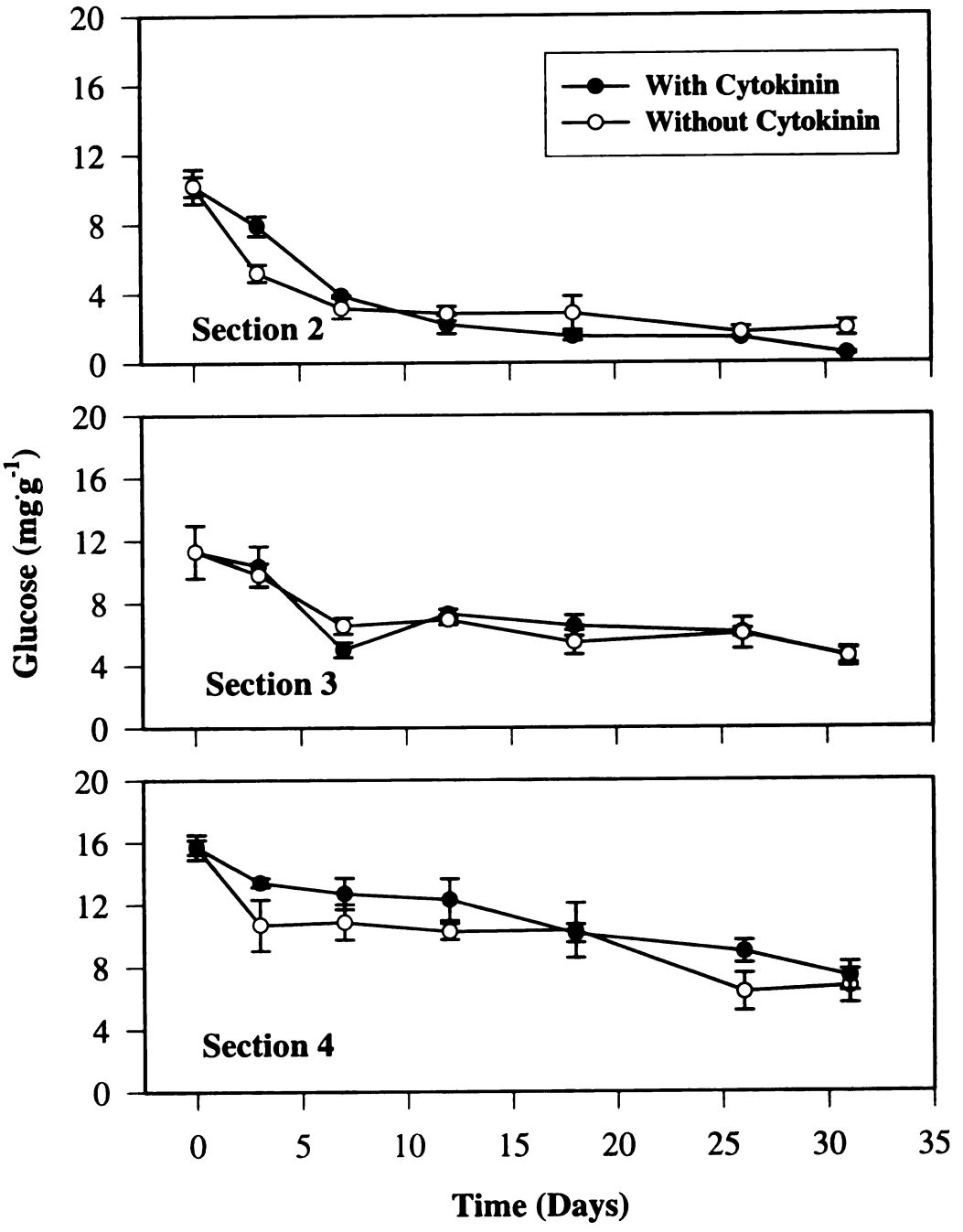

Figure 5.5. Changes in glucose content (fresh mass basis) in asparagus spears without 45 mm long tip segment, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

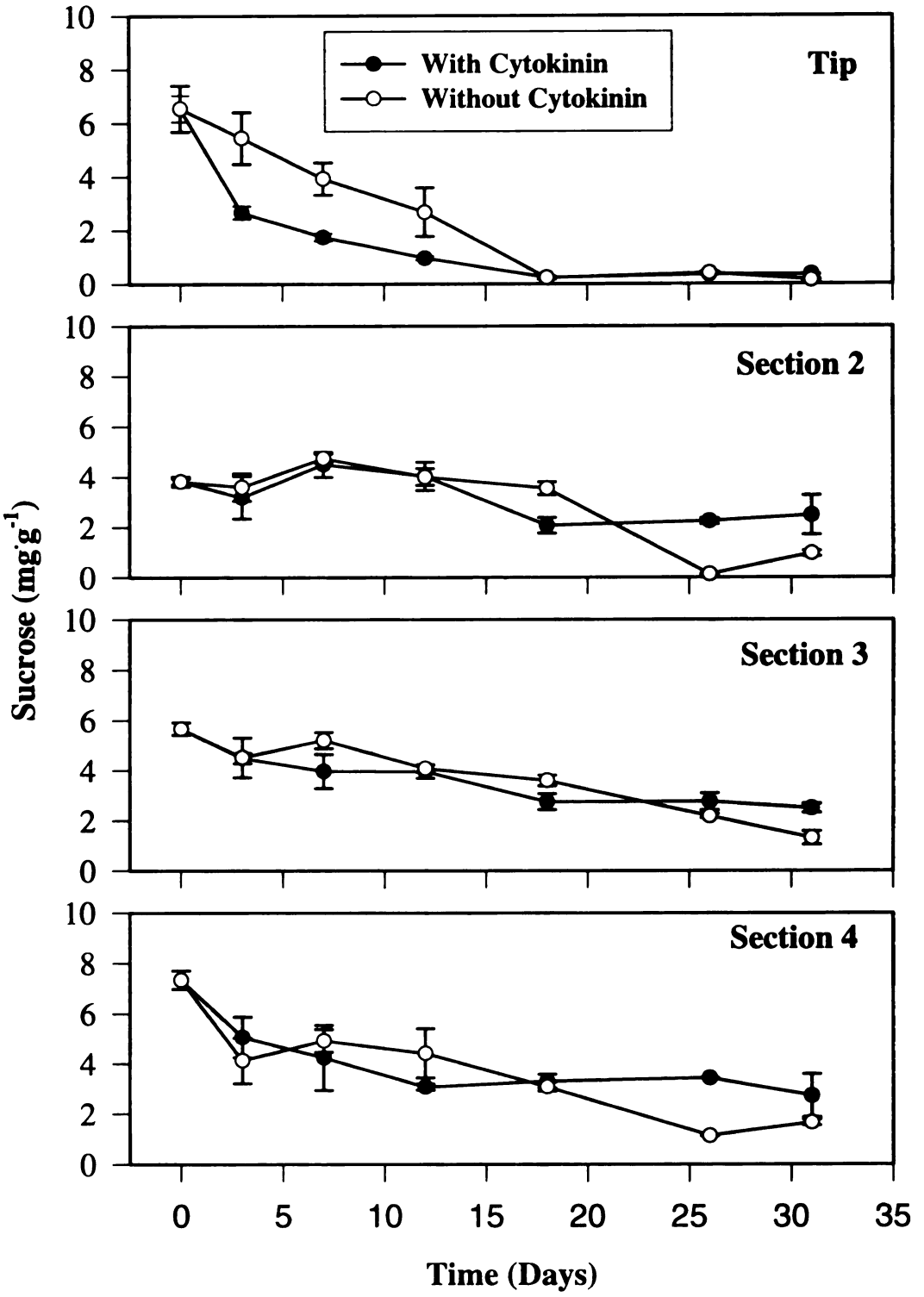

Figure 5.6. Changes in sucrose content (fresh mass basis) in asparagus spears with tips, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

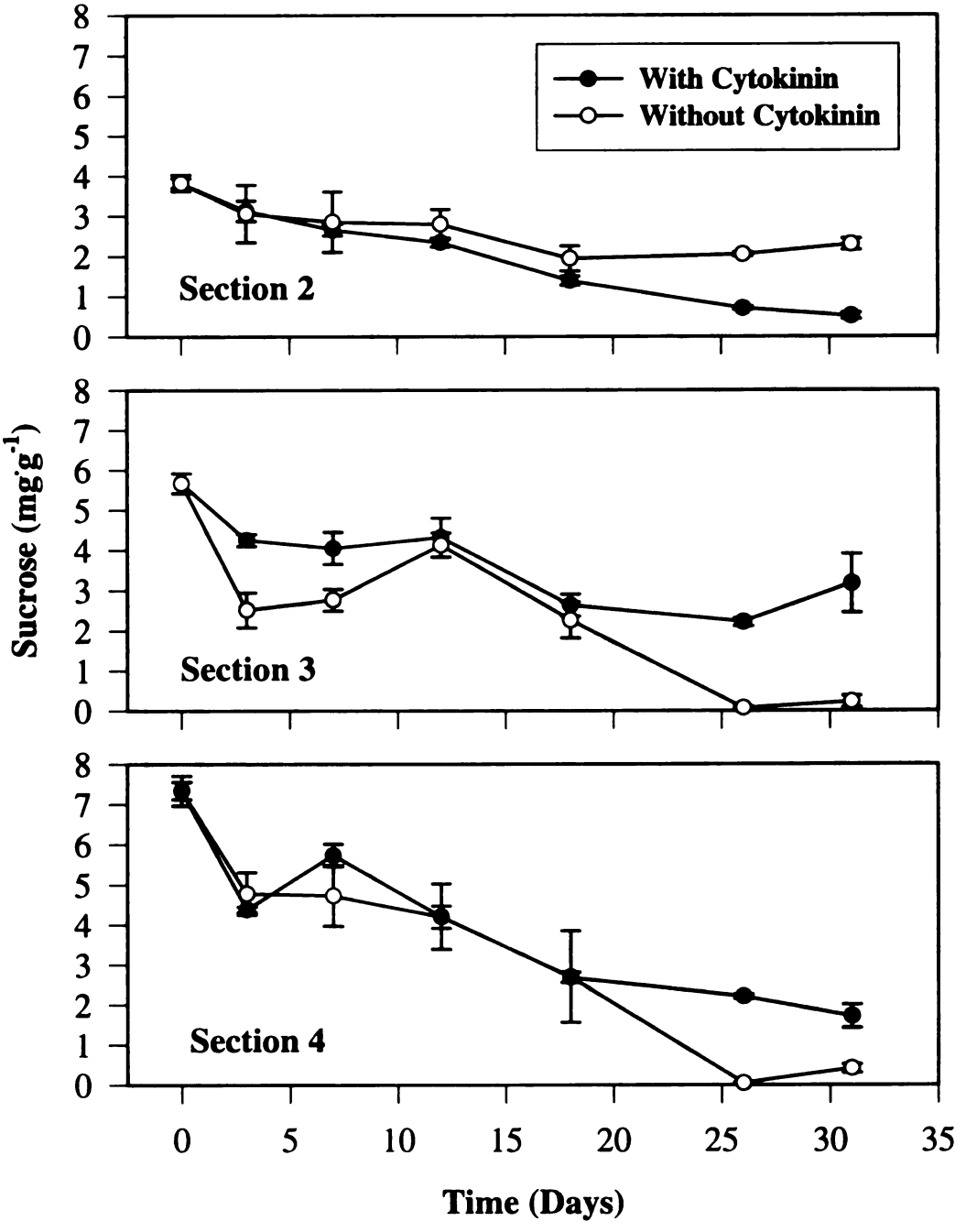

Figure 5.7. Changes in sucrose content (fresh mass basis) in asparagus spears without 45 mm long tip segments, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

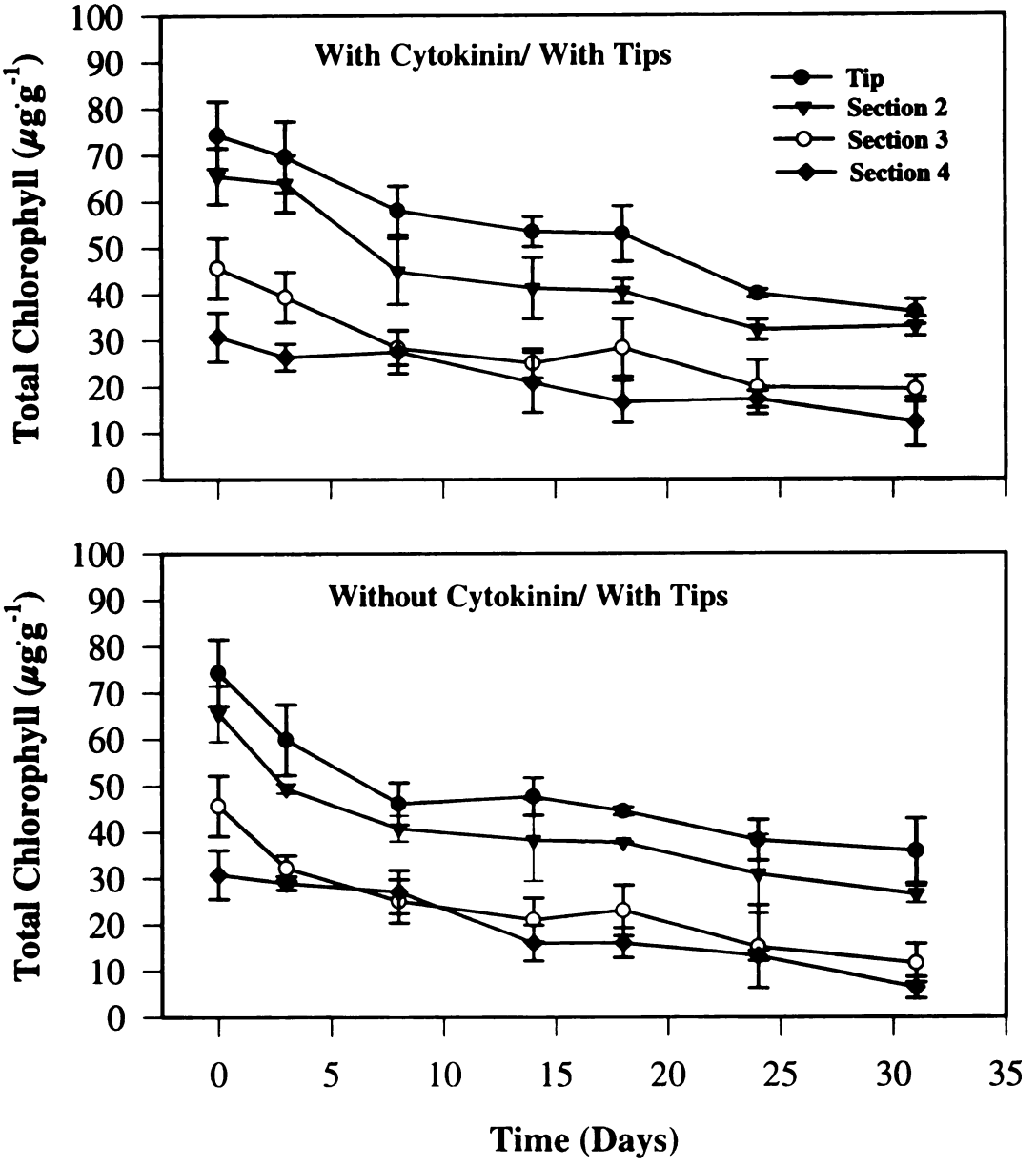

Figure 5.8. Changes in total chlorophyll content (fresh mass basis) in asparagus spears with tips, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

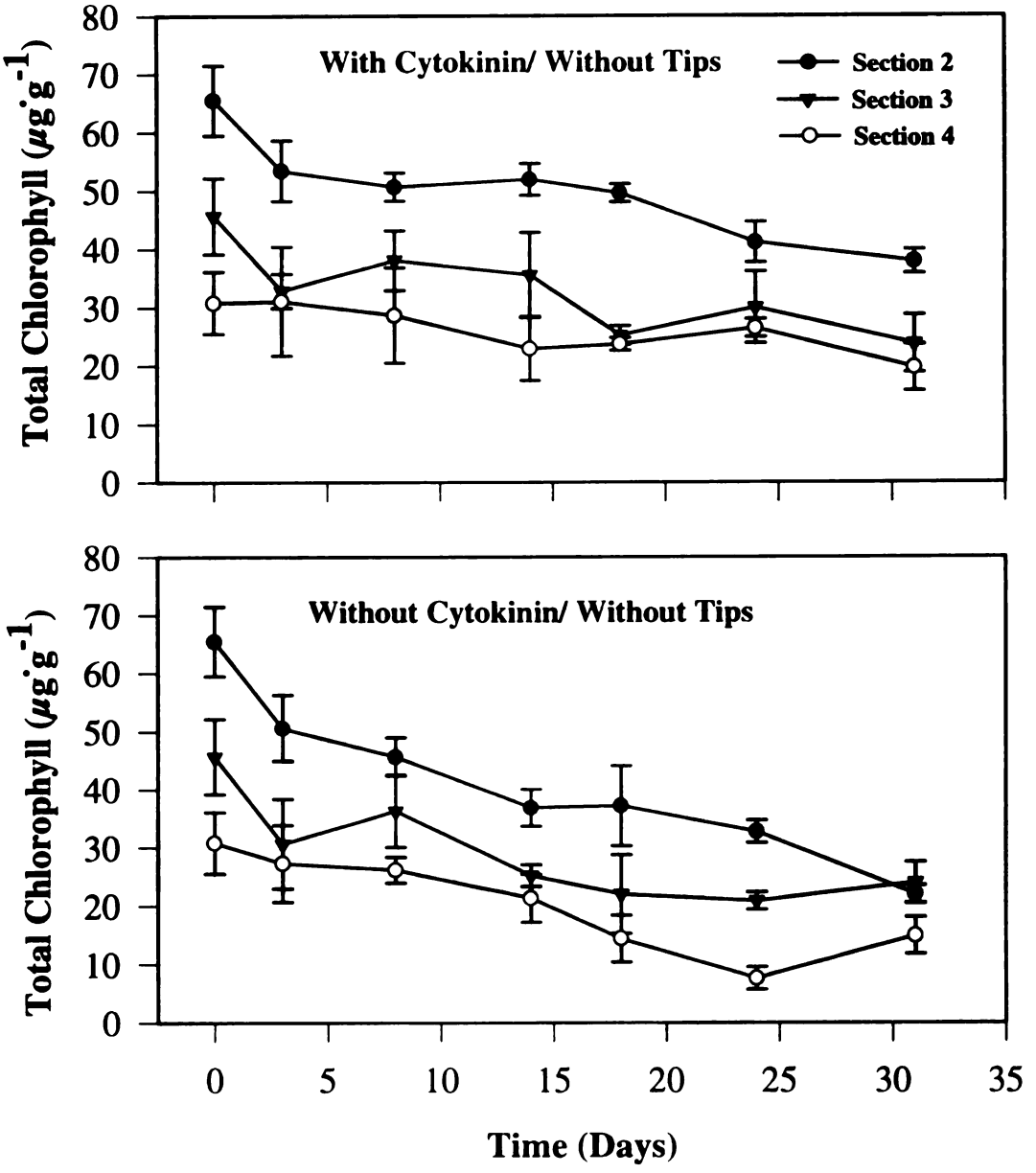

Figure 5.9. Changes in total chlorophyll content (fresh mass basis) in asparagus spears without 45 mm long tip segments, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=3).

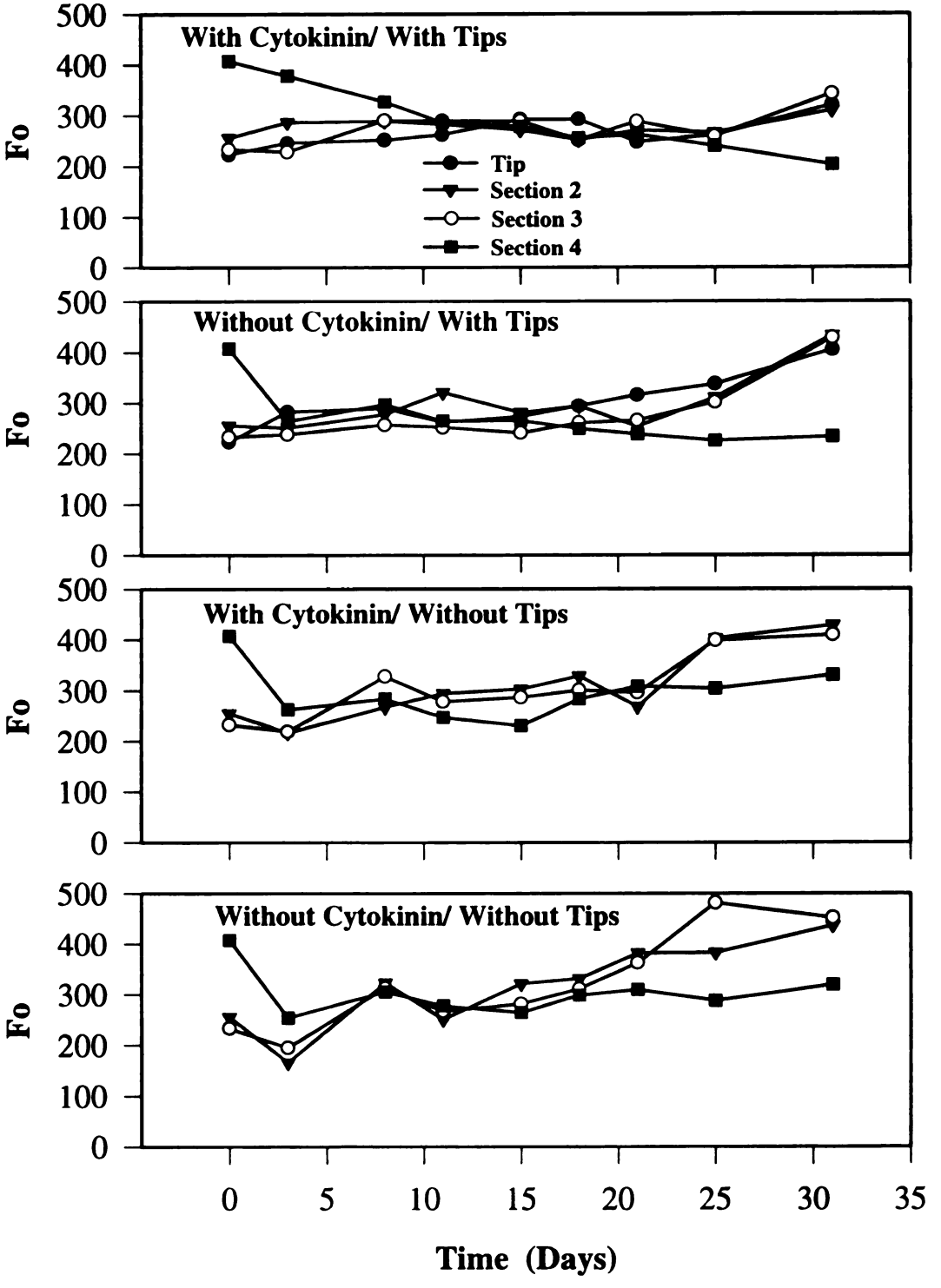

Figure 5.10. Changes in the basal chlorophyll fluorescence (Fo) in asparagus spears with and without 6-BAP, with and without 45 mm long tip segments, during storage at 1 °C. Bar = ± SE (n=3).

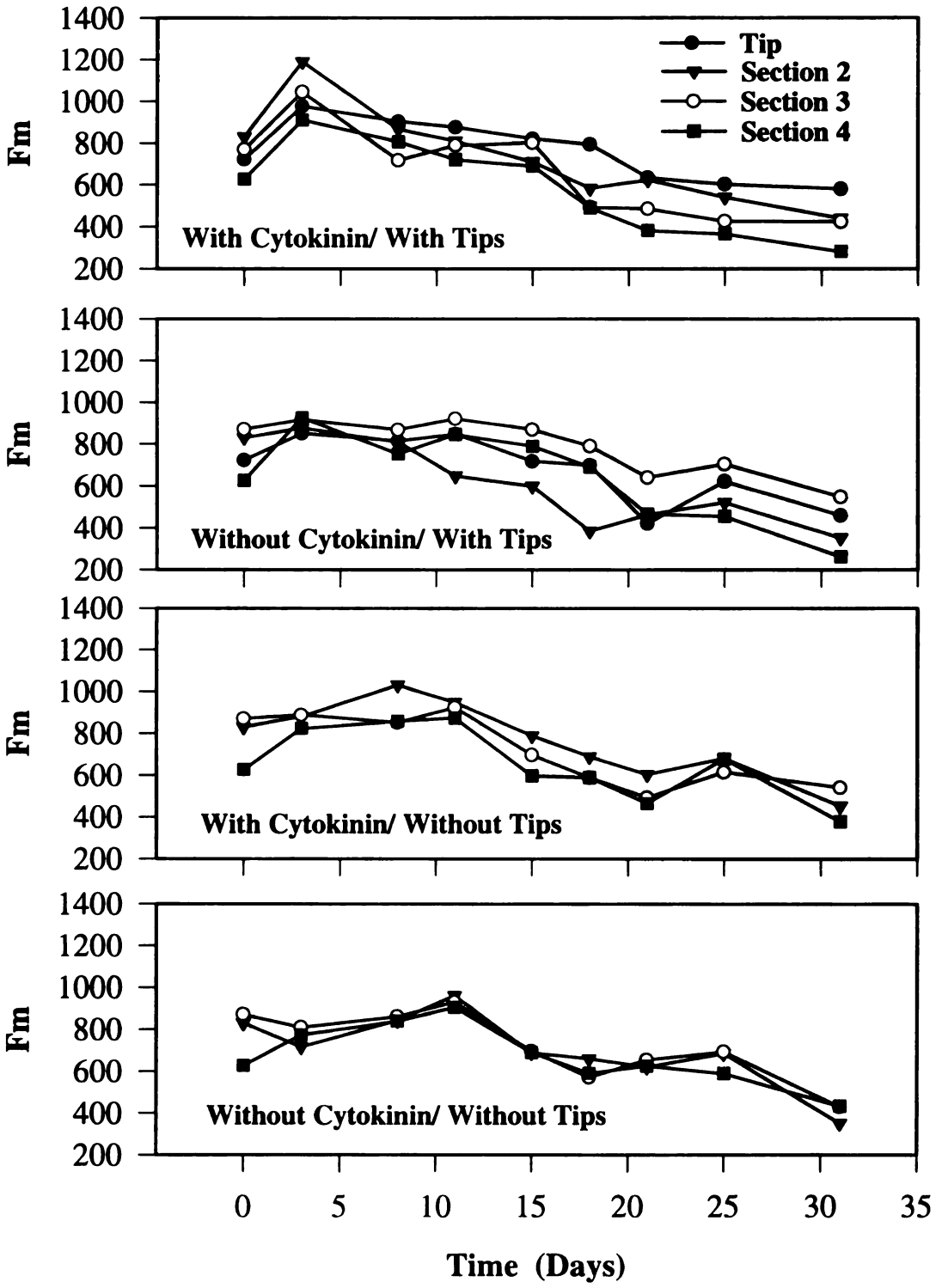

Figure 5.11. Changes in the maximum chlorophyll fluorescence (Fm) in asparagus spears with and without 6-BAP, with and without 45 mm long tip segments, during storage at 1 °C. Bar = ± SE (n=3).

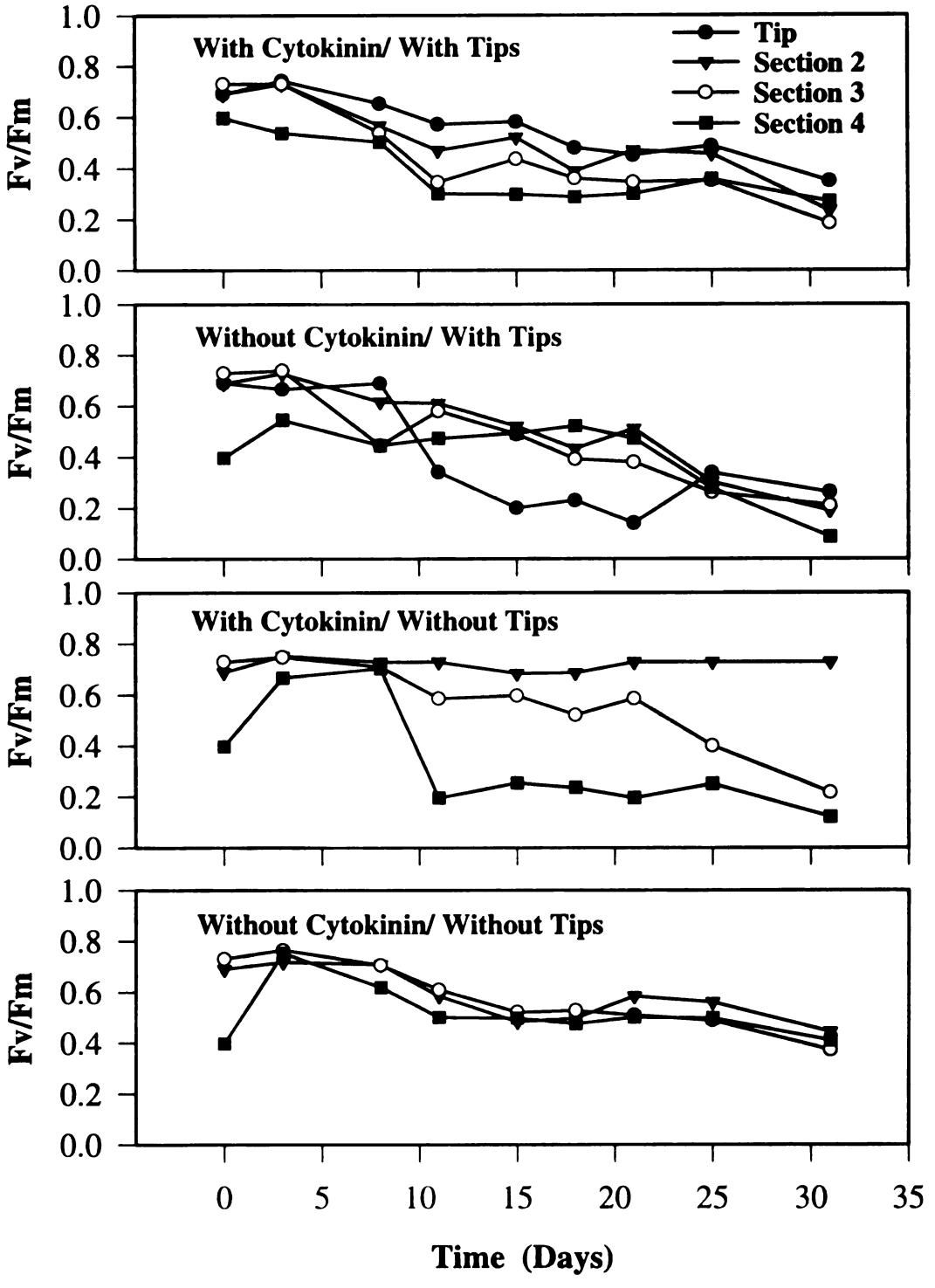

Figure 5.12. Changes in the quantum yield maximum (Fv/Fm) in asparagus spears with and without 6-BAP, with and without 45 mm long tip segments, during storage at 1 °C. Bar = ± SE (n=3).

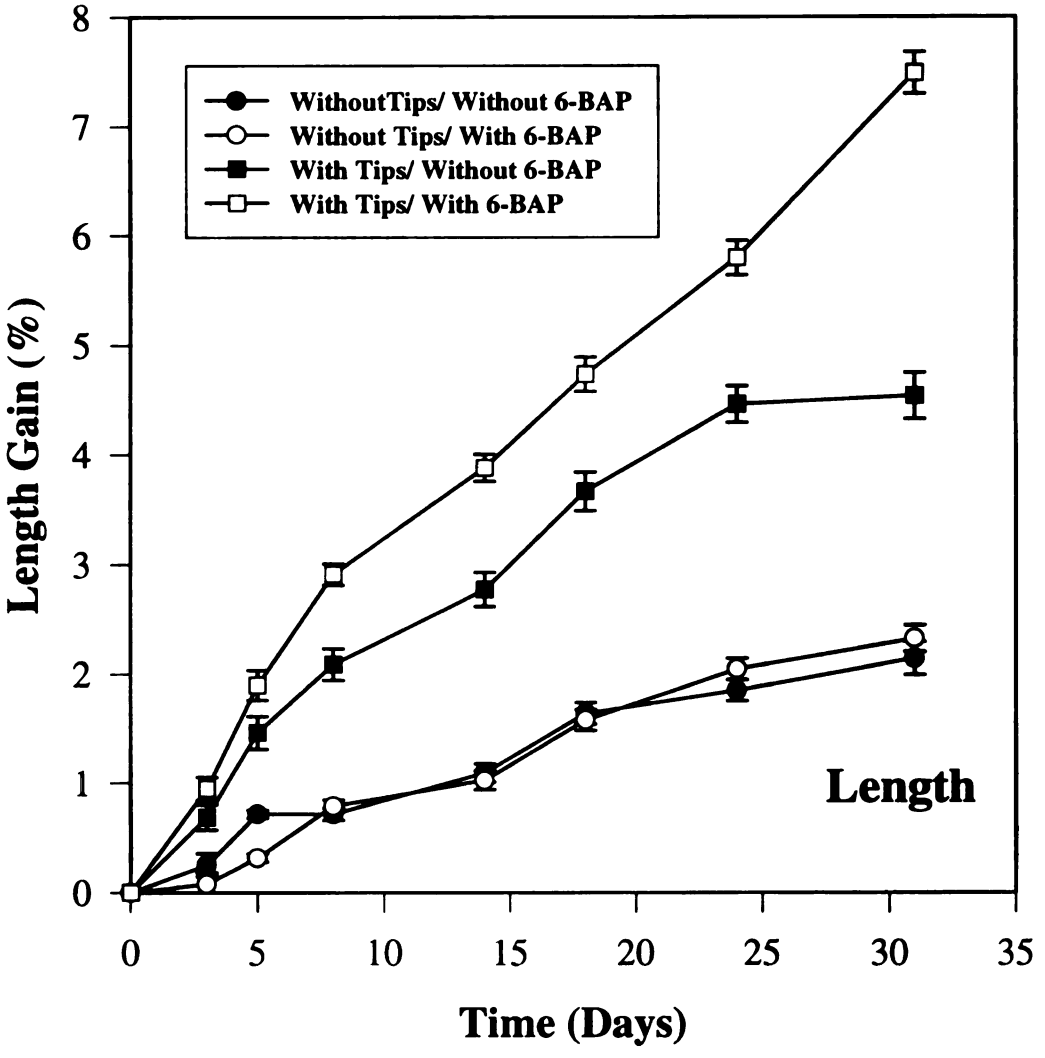

Figure 5.13. Changes in length relative to the initial storage time in asparagus spears with and without 45 mm long tip segments, with and without 6-BAP, during storage at 1 °C. Bar = ± SE (n=10).

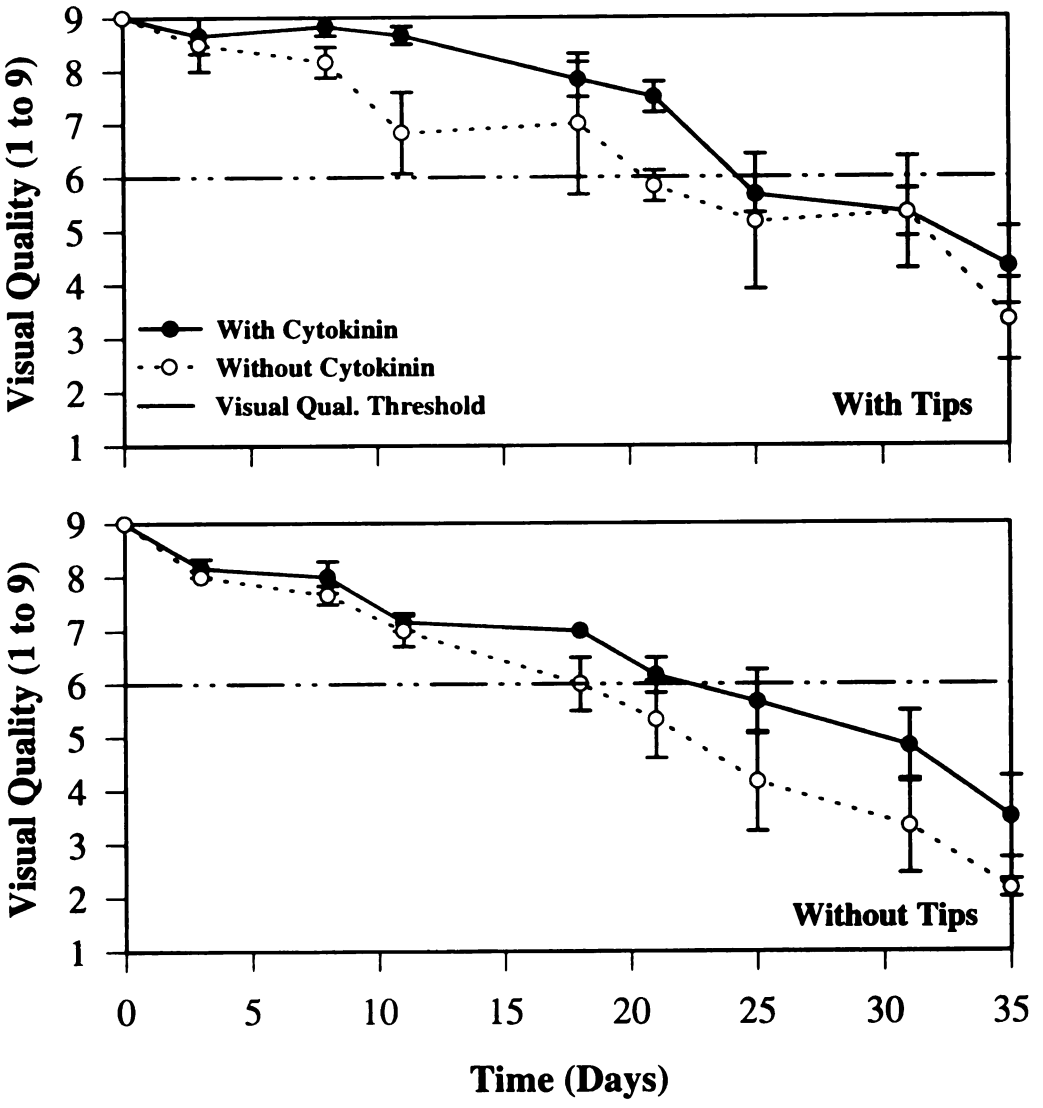

Figure 5.14. Changes in visual quality in asparagus spears with and without 6-BAP, with and without 45 mm long tip segments, during storage at 1 °C. Value 6 represents the visual quality threshold. Bar = ± SE (n=3).