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EFFECTS OF CONTROLLED ATMOSPHERE STORAGE AND MODIFIED ATMOSPHERE PACKAGING ON CHINESE MUSTARD

By

HONG WANG

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ABSTRACT

EFFECTS OF CONTROLLED ATMOSPHERE STORAGE AND MODIFIED ATMOSPHERE PACKAGING ON CHINESE MUSTARD

BY

HONG WANG

Chinese Mustard (Brassica Campestris Chinensis group) was stored in the following controlled atmosphere conditions at 10 °C: 1) air (control), 2) 3% oxygen, 3) 5% carbon dioxide in air and 4) 3% oxygen plus 5% carbon dioxide.

A study of the ultrastructure of leaf tissue showed that during leaf senescence there was a progressive degeneration of the membrane structure of the grana of the chloroplast accompanied by the appearance of globules of lipid material and disappearance of starch grains. Loss of chlorophyll and protein degradation were observed during leaf senescence. A controlled atmosphere of 5% CO_2 plus 3% O_2 maintained chloroplast grana membrane structure and well-defined mesophyll cells for up to 4 weeks storage. Both 5% CO_2 in air and 3% O_2 plus 5% CO_2 maintained the highest chlorophyll levels compared to 3% O_2 alone or air (control).
High CO_2 (5%) plus low O_2 (3%) reduced the loss of ribulose-1,5-bisphosphate carboxylase activity and protein degradation.

Polypeptide profiles were compared among leaf tissues from the various CA treatments storage by SDS-PAGE. There was a decrease in band size for some polypeptides from 3% O_2 treatment and these bands were further diminished in the control. Loss of bands were observed in the control, correlated with the appearance

of two new bands.

These results suggest that CA may delay leaf senescence by maintaining compartmentation of chloroplast and reducing chlorophyll loss and protein degradation.

Studies on effect of Modified Atmosphere packaging (MAP) on extending shelf-life of Chinese mustard were conducted. It was found that MAP could reduce weight loss of Chinese mustard. MAP with less permeability could extend shelf-life of Chinese mustard in terms of reducing trim loss, retarding chlorophyll degradation and keeping freshness. As storage temperature increased, the steady state concentrations of O_2 and CO_2 within packages decreased and increased respectively. Respiration of Chinese mustard was more sensitive to temperature than was permeability of the package. Respiration, weight loss and shelf-life of Chinese mustard were temperature dependant.

MAP could be an efficient, low cost and energy saving technology for extending shelf-life of Chinese mustard.

To my son, Paul Fang

To my husband and best friend, Guowei Fang

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LITERATURE REVIEW

GENERAL ASPECTS OF LEAF SENESCENCE

There are a number of definitions of leaf senescence (Huber, 1987). The narrow definition given by Thomas and Stoddard (1980) will be used in this thesis: "Senescence is a series of events concerned with cellular disassembly in the leaf and the mobilization of the materials released". Initiation of senescence signifies a transition in leaf function from assimilation to remobilization (Stoddart and Thomas, 1982). Five different types of leaf senescence have been described (Simon, 1967). In this paper we are concerned with harvested plants and shoots which do not readily fit any of these five types.

Leaf senescence is a developmental change that leads to a loss of leaf function and ends in death of the leaf. In leaves, senescence is marked by a decline in chlorophyll, protein, RNA, DNA and growth promoters and by an increase in hydrolytic enzymes and growth inhibitors (Thomas and Stoddart, 1980; Woolhouse,1987; Brady, 1988; Sacher,1973). Although these symptoms of senescence are well known, the mechanism that regulates senescence is little understood.

Cell deterioration and death during senescence follow a characteristic cytological pattern (Shaw and Manocha, 1965; Barton, 1966; Butler and Simon, 1971; Thomson and Platt-Aloia, 1987). These changes are:

1) A reduction in the number of ribosomes, and the beginning of the chloroplast-to-chromoplast transition (Barton, 1966; Hurkman, 1979), Starch granules disappear early and swell in some cases (Dennis, et al., 1967; Colquhoun et al.,1975) but not in others (Mlodzianowski and Ponitka,1973).

2) The thylakoids become less dense and osmiophilic granules increase in number and density (Barton, 1966; Dennis et al., 1967; Mlodzianowski and Ponitka, 1973; Hurkman,1979).

3) The cisternal endoplasmic reticulum becomes tubular and vesiculate. This is followed by breakdown and subsequent disappearance of the endoplasmic reticulum and Golgi apparatus (Barton, 1966).

4) The disintegration of the vacuolar membrane occurs late in senescence (Barton, 1966), generally before the cellular organelles completely disintegrate.

5) The nucleus is usually stable until relatively late. When the nuclear membrane becomes vesiculate and breaks down, the chromatin also disappears (Thomson and Platt-Aloia, 1987).

6) Mitochondria show some early changes in size and morphology, but are still present and functional at a late stage. Death is usually the result of, or at least preceded by, the breakdown of the plasmalemma (Thomson and Platt-Aloia,1987).

Two important points are: 1) the integrety of the membrane system which maintains compartmentation of the cell is closely associated with senescence (Thomas, 1987; Dalling, 1987), and 2) the inducer of senescence appears to be located in the cytoplasm (Barton, 1966).

It may be that senescence is dependent upon a continuing dynamic state of macromolecules in the cells (Brady, 1988). Senescence is a sequential process during which a developmental threshold is crossed and the syndrome becomes irreversibly established (Stoddart and Thomas, 1982).

Senescence has been a concern of plant physiologists for many years. There are a

number of reviews of leaf senescence (Osborne, 1967; Brady, 1973; Beevers, 1976; Nooden and Leopold, 1978; Thomas and Stoddard, 1980; Thimann, 1980; Sexton and Woolhouse, 1984; Woolhouse, 1967, 1982, 1983, 1984, 1986; Kelly and Davies, 1988; Nooden and Leopold, 1988). These reviews rarely mention any leafy vegetables, but are devoted mainly to the senescence of cereal leaves, tobacco, some legumes and other scientifically suitable systems. The number of species considered is very limited (Kelly and Davies, 1988), making the formulatuon of a broad perspective difficult. The specific lack of information and research on senescence of economically important leafy vegetables has been made clear (Lipton, 1987). Though some work has been done on excised leaves, these have generally been cereals. There is a large body of literature dealing with attached cereal leaves because of their importance economically and agronomically. These studies can provide direction for research but the experimental process would be expected to be greatly modified under the conditions experienced in the postharvest handling chain. The work from fruit and flower senescence is used in predicting leaf senescence, but the different tissue responses would be a significant limiting factor in interpretation (Goldschmidt, 1986; Brady, 1988). Some limited work has been done on temperate leafy vegetables, especially lettuce (Lipton, 1987). This, however, has not addressed the mechanism of senescence, but of the response to postharvest treatments. In the postharvest situation, the objective is to maintain leaf components, reduce remobilization and keep changes at molecular, organelle, cellular and tissue levels to a minimum. However, the effect of postharvest conditions on the senescence of leafy vegetables and the control of this mechanism is one of the least studied areas of senescence.

How do postharvest storage and handling procedures modify the process of senescence? The answer to this question would provide an understanding of senescence and would have a number of practical benefits to postharvest senescence.

Chinese Mustard or Pak Choi (Brassica Campestris, chinensis group) is a very important vegetable in China and Asian and is becoming popular in areas with large Asian and Pacific island populations in the U.S.. There is little research on senescence of Chinese mustard under postharvest conditions. Lack of information on postharvest handling and senescence control greatly limits the market potential of this commodity and results in great waste. An understanding of the postharvest physiology and senescence of Chinese mustard would enable better procedures to be developed to store similar leafy vegetable crops. This would allow supply variation to be reduced and quality to be maintained.

RIBULOSE BISPHOSPHATE CARBOXYLASE AND LEAF SENESCENCE

Ribulose bisphosphate (RUBP) carboxylase/oxygenase (EC 4.1.139) is a major protein component of the chloroplast stroma (> 50%). The carboxylase protein is composed of eight large and eight small subunits differing in their size and amino acid composition. The large subunit has a molecular weight of about 55,000 and the small subunit has a weight of about 15,000 (Baker et al., 1975). The large subunits are coded in the chloroplast genome (Kung, 1976) and are synthesized within the chloroplast on chloroplast ribosomes (Blair and Ellis, 1973). The small subunits are coded within the nuclear genome (Kung, 1976) and are synthesized outside the chloroplast on cytoplasmic ribosomes and transported into the chloroplasts for assembly into the whole enzyme (Gray and Kekwick, 1974; Smith and Ellis, 1979;

Speirs and Brady, 1981). The coordination of large and small subunits for ribulose bisphosphate carboxylase synthesis, for example, could be mediated by signals perceived in one subunit and transduced by a secondary signal to the other (Rodermel et al., 1988). The large subunit contains the active site . The small subunits are thought to have a regulatory function (Robinson and Walker, 1981).

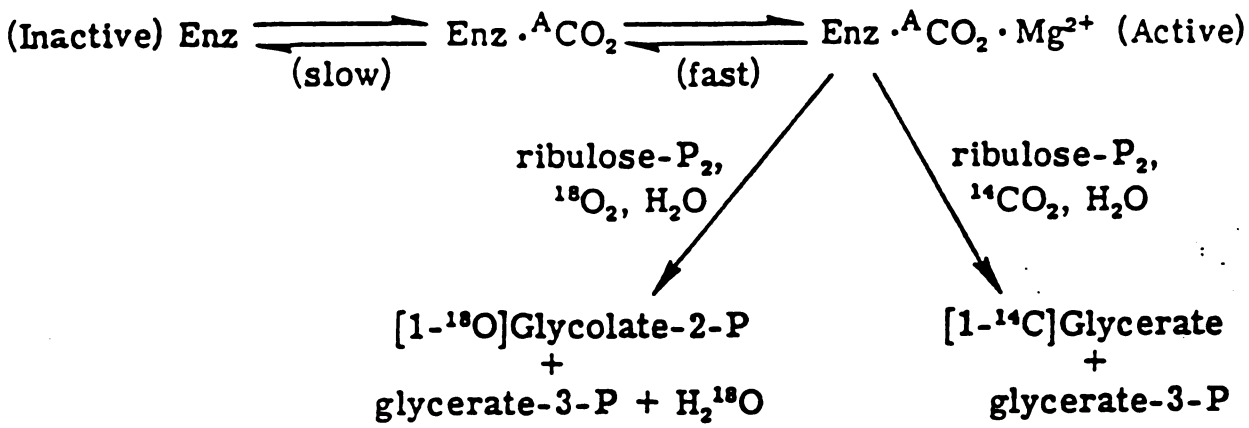
About 90-95% of the total soluble protein lost during senescence is carboxylase (Woolhouse, 1967; Peterson and Huffaker, 1975). The synthesis of carboxylase declines in proportion to total protein synthesis (Woolhouse, 1984) in leaves of wheat (Brady and Tung, 1975), Perilla frutescens (Kannangara and Woolhouse, 1968), cucumber (Callow, 1974) and poplar (Dickman and Gordon, 1975) during aging and senescence. The cause of this decreased RUBP carboxylase is not entirely clear; most studies reported a decrease in this enzyme but in tobacco, cucumber and wheat, inactivation as well as loss of the enzyme appears to be involved since the specific activity of the enzyme is also reported to decrease. Turnover studies have been reported for RuBP carboxylase and in Perilla (Woolhouse, 1967) and barley (Peterson and Huffaker, 1975), This protein is not turned over once expansion of the leaf is completed while in Zea Mays turnover of the RuBP carboxylase continues in the mature leaf, although with degradation exceeding synthesis in the late stages.

During aging, there is a decreasing abundance of translatable mRNA for both the small and large subunits relative to most other mRNA (Speirs and Brady, 1981). This decline in synthesis of mRNA closely resembles the in vivo decline in synthesis of subunits (Speirs and Brady, 1981; Miziorko and Lorimer, 1983). Therefore, ribulose 1,5-bisphosphate carboxylase becomes a declining proportion of total protein

synthesis.

Ribulose 1,5-bisphosphate (ribulose-P2)carboxylase/ oxygenase is a bifunctional enzyme that catalyzes the addition of CO_2 or O_2 to C-2 of ribulose-2P. The products of the carboxylase reaction are two molecules of D-glycerate-3-P, and this enzyme activity is systematically classified as 3-phospho-D-glycerate carboxyl-lyase (dimerizing). The carboxylase activity can be assayed radiochemically by the rate of formation of acid-stable ^{14}C product ([1-^{14}C] glycerate-3-p) formed from the reaction of RuBP with [^{14}C]O_2 (Wishnick and Lane, 1971; Paulsen and Lane, 1966).

An appreciation of the protocols used in properly assaying ribulose-P2 carboxylase/oxygenase activity requires an understanding of the manner in which various forms of the enzyme interconvert. The forms of ribulose-P2 carboxylase/ oxygenase are shown in the following scheme:



The first two reactions of the scheme are referred to as activation and involve the slow, reversible addition of CO_2 (A CO_2= activator CO_2) to a lysal residue on the enzyme to form a carbamate. This is followed by a rapid, reversible interaction of the carbamate form of the enzyme with Mg^{2+} to form the active, ternary complex (Lorimer, 1981). The adjective, "slow", used in describing the formation of the

enzyme-ACO_2 complex, is meant to indicate that the rate of formation and breakdown of this binary complex is much slower than the rate of catalysis. The multiple effects of CO_2 on enzyme activity require careful consideration. In addition, a different molecule of CO_2 (sCO_2= substrate CO_2) is used as the substrate for the carboxylase reaction. sCO_2 and O_2 are competitive inhibitors with respect to the oxygenase and carboxylase reactions. Three factors which tend to push the equilibrium of the reaction to the direction of activated enzyme are CO_2, Mg^{2+} and alkaline pH. Thus, to ensure that one measures the maximum rate of catalysis, it is essential to optimize both the conditions of catalysis (substrate concentration, pH, etc.) and to ensure that each reaction is initiated with fully activated enzyme.

PROTEIN AND LEAF SENESCENCE

The protein content of the leaf declines progressively as a result of breakdown of protein to amino acids and amides during senescence (Woolhouse, 1967; Thimann, 1987; Thomas and Hilditch, 1987). It has been observed that external factors that decrease the rate of protein degradation also slow down senescence. Inhibitor studies in general have shown that leaf senescence is insensitive to transcription inhibitors (Thomas, 1975), delayed by translation inhibitors (Martin and Thimann, 1972; Thomas, 1975; Makovetzki and Goldschmidt, 1976; Peterson and Huffaker, 1975) and not affected by post-transcriptional processing inhibitors (Paranjothy and Wareing, 1971; Takegami and Yoshida, 1975; Thomas and Stoddard, 1980).

CHLOROPHYLLS AND LEAF SENESCENCE

Yellowing is the most visible symptom of senescence in green leafy vegetables and results from a loss of chorophyll. The rate of chlorophyll loss depends greatly on

maturity of the leaf, on temperature, the atmosphere and the species (Hendry et al., 1987).

A rapid decline in photosynthesis rate per unit leaf area occurs before appreciable loss of chlorophyll and is not ascribed to chlorophyll loss (Hernandez-Gil and Schaedle, 1973; Misr and Meena, 1986). This decline is associated with the loss of RuBP carboxylase protein and the corresponding loss of activity (Wittenbach et al., 1980). The onset and rate of decline in chlorophyll may or may not be coupled with the decline in photosynthetic rate (Ford and Shibles, 1988).

CONTROLLED ATMOSPHERE STORAGE / MODIFIED ATMOSPHERE PACKAGING AND LEAF SENESCENCE

Since studies on the use of controlled atmosphere (CA) on tomato were reported by Kidd and West in 1932, five years after their report on its use with apples, there have been many studies conducted (Isenberg, 1979). The principal benefit that one can expect from CA /MA storage is that the product so stored will maintain its freshness and eating quality for significantly longer period than it would if stored at the same temperature in air. The owner of such produce expects to market it at the time when both the quantity of available product is low, and the quality of the competing product not stored in CA/MA is poor. Used properly, CA/MA can significantly supplement proper holding temperature by maintaining the quality of selected fruits and vegetables for longer durations than normally encountered in normal air. Extending shelf-life while maintaining desirable market quality opens the door to new overseas markets and allows for increased flexibility in meeting market demands.

The basic principle of CA/MA storage is the imposition of an abnormal external environment for the purpose of lowering the rate of biochemical processes, retarding senescence and associated physiological and biochemical changes. The term "controlled atmosphere" (CA) and "modified atmosphere" (MA) mean that the atmospheric composition surrounding a perishable product is different from that of normal air. Both involve manipulation of carbon dioxide, oxygen, and nitrogen level; however, other gases such as carbon monoxide, ethylene, and humidity are sometimes included. MA differs from CA in how precisely gas partial pressures are controlled; CA is more precise than MA. The high degree of atmospheric regulation associated with CA is capital intersive and expensive to operate and thus is more appropriate for commodities that are amenable tol long-term storage such as apple, cabbage, kiwifruit, and pear (Kader et al., 1989). MA storage implies a lower degree of control of gas concentrations. Typically, initial atmospheric conditions are established for a transient period, and the interplay of the commodities' physiology and the physical environment maintain those conditions within broad limits (Zagory and Kader 1988). Hypobaric storage is a type of CA storage in which a product is held under a partial vacuum (Dilley, 1977). Figure 1 illustrates how manipulation of the environment can take place in a CA system (Brecht, 1980). In this model, three barriers are shown: the commodity itself, the package, and the storage room or transit vehicle. A dynamic equilibrium exists between the endogenous gases produced at various centers of enzymes action in the cells and the exogenous gases surrounding the commodity. The balance between exogenous and endogenous components influences the rate of diffusion into and out of the commodity and hence the

resultant atmosphere within the commodity (Brecht, 1980).

Numerous factors have been shown to influence the effects of CA/MA on vegetables and fruits: the sensitivity of the vegetable or fruit, maturity stage at harvest, the concentration of CO_2 and O_2 in the atmosphere, the temperature and the length of exposure time. The commodity itself can differ markedly in response to elevated CO_2 or lowered O_2. Some cultivars of crisphead lettuce shows signs of brown stain (a form of CO_2 injury) when subjected to CO_2 levels of 1% or above, whereas Romaine lettuce can tolerate levels up to 12% (Lipton, 1977). Cultivars differ greatly in their response to elevated CO_2 and /or lowed O_2. Brecht et al., (1973) subjected 11 cultivars of crisphead lettuce to elevated CO_2 and noted significant differences in incidence and severity of brown stain.

According to Burton (1974), the most important factor influencing the composition of the gaseous phase within the intercellular space of the tissue is the resistance to diffusion by the outer intergument plus the rate of O2 consumption and CO2 output of the tissue. The differential sensitivity of various vegetables to CO2 may be related to different internal levels of CO2 and O2 which change because of differences in respiration rate, outer integument characteristics (presence or absence of stomata, cuticle thiakness) and internal gaseous volume (Herner, 1987).

Since respiration generally is a good indicator of metabolic rate, reducing respiration can be a means of slowing metabolism and extending storage life. The influence of CA/MA on respiration rate and other basic catabolic processes of stored fruits and vegetables has been documented in several reviews (Isenberg, 1979; Smock, 1979; Burton, 1978). The effect of CA/MA on respiration is dependent on the

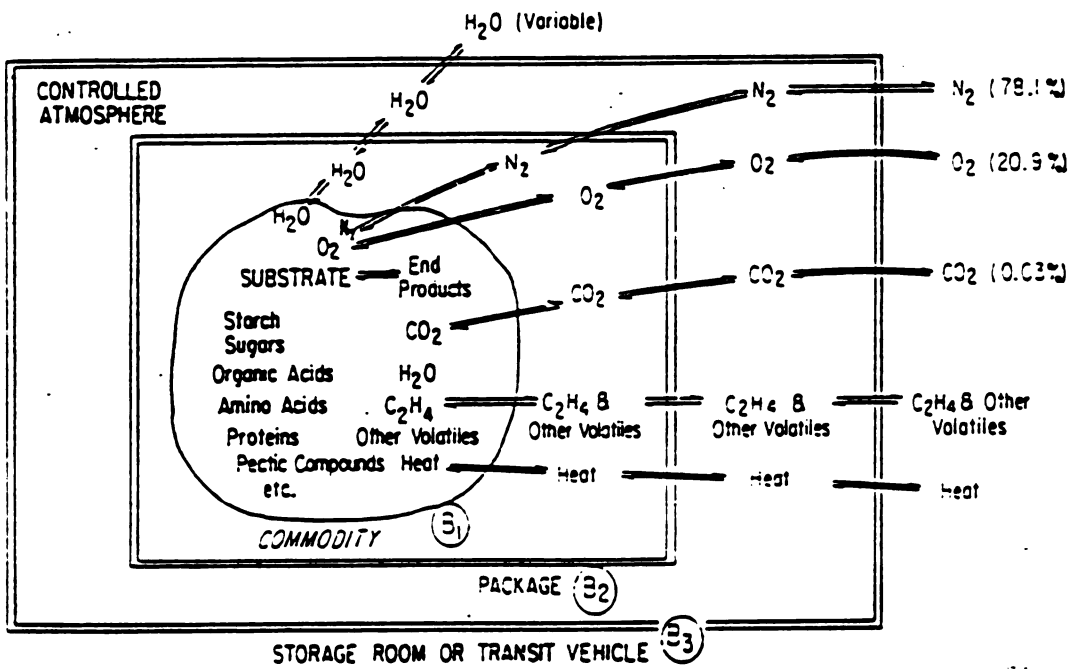


Figure1. Model of a commodity with a controlled atmosphere environment, illustrating gas exchange across three barriers-the commodity itself (B1), the package (B2), and the storage room or the vehichle (B3)(Brecht,1980).

plant material itself and on the O2 concentration gradient that develops between the centers of metabolic action and the outer integument of the plant materials. Lowering O_2 and elevating CO_2 reduce respiration rate, but a minimum of about 1-3% O_2 depending on the commodity is required to avoid a shift from aerobic to anaerobic respiration. If the concentration of CO_2 is too high, injury may result (Herner, 1987; Weichmann, 1977a; 1977c; 1980).

A significant benefit of CA/MA is that chlorophyll loss or degradation can be delayed. Elevated CO_2 and reduced O_2 have been shown to reduce loss of chlorophyll in green bean (Groeschel,1964; Groeschel et al.,1966; Nelson, 1965), crisphead lettuce (Singh et al., 1972), broccoli (Leberman et al.,1968;Lipton and Harris, 1974; Nelson, 1965), Brussels sprouts (Eaves and Forsyth, 1968; Lyons and Rappaport, 1962), cabbage (Isenberg and Sayles, 1969), green asparagus (Wang et al., 1971), Chinese cabbage (Wang, 1983; Weichmann,1977b), green cabbage (Isenberg and Sayles, 1969; Geeson and Browne, 1979; 1980), savoy cabbage (Stoll,1974), and leek (Hoftun,1978; Kurki,1979; Weichmann,1979).

Aging of vegetative tissue by ethylene exposure can induce physiological disorders. By reducing the O_2 level to between 2 and 6 %, the incidence of russet spotting in crisphead lettuce can be effectively reduced because of reduced ethylene production (Ryall and Lipton, 1972).

In summary, the following general statements can be made regarding leaf senescence:

1). Leaf senescence is apparently programmed and translational control may be a major factor, however, little is known about its mechanism.

2). Cellular compartmentation, maintained by the membrane system is essential to normal metabolism. Therefore, any factor which leads to the disintegration of membranes enhances leaf senescence, whereas those which can maintain integrity of the membrane system may delay senescence.

3). Limiting the O2 supply and elevating the CO2 level decrease the rate of leaf senescence.

Much research has been directed toward determination of optimum CA/MA conditions for a large number of fruits and vegetable and specific cultivars of each commodity, but less of these reports dealt with the mode of action of reduced oxygen and elevated carbon dioxide concentration, i.e., the biochemical and physiological basis for CA/MA effects on fruits and vegetables.

Generally, the effect of reduced Oxygen and /or elevated carbon dioxide on reducing respiration rate has been assumed to be the primary reason for the beneficial effects of CA/MA on fruits and vegetables. This is an oversimplification. Since postharvest deterioration of fresh produce can be caused by many factors in addition to high respiration rate, including: metabolic changes (biochemical changes associated with respiratory metabolism, ethylene biosynthesis and action, and compositional changes); physical injury, physiological disorders; pathological breakdown and growth and development (anatomical and morphological changes). Such information will no doubt help in expanding the use of CA or MA during transport and storage of perishable commodities.

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CHAPTER ONE

EFFECT OF CONTROLLED ATMOSPHERE STORAGE ON CHINESE MUSTARD

INTRODUCTION

Chinese mustard or Pak Choi (Brassica Campestris, Chinensis group) is a very important vegetable in China and Asia and is becoming popular in areas with large Asian and Pacific island populations in the U.S.. Chinese Mustard is a biennial vegetable crop having some of the characteristics of swiss chard growth habit. The leaves are long, dark green and oblong or oval; it does not form a solid head (Figure 2). Lack of information on postharvest handling and senescence control greatly limits the commercial use of this commodity and causes great waste. An understanding of the postharvest physiology and senescence of Chinese mustard would enable better procedures to be developed to store similar leafy vegetable crops. This would allow supply variation to be reduced and quality to be maintained.

Much research has been directed toward determination of optimum CA conditions for a large number of fruits and vegetables and specific cultivars of each commodity, but few of these reports have dealt with the mode of action of reduced oxygen and elevated carbon dioxide concentration, i. e., the biochemical and physiological basis for CA effects on fruits and vegetables.

The objectives of this research were :1). to determine the postharvest translational changes which occur normally and compare this with the changes in tissues whose senescence process has been modified by CA,

2). to determine the changes in chlorophyll, trim loss, RuBP carboxylase activity in

the leaf tissues of Chinese mustard subjected to different storage atmospheres. 3).and to observe the ultrastructural changes of leaf cells of Chinese mustard exposed to various storage atmospheres.

The controlled atmospheres chosen were air (control), 3% O_2, 5% CO_2 in air and 5%CO_2 plus 3% O_2. A temperature of 10°C was used because 1) refrigeration is expensive in developing countries and it was thought that a moderate temperature plus controlled atmosphere might provide a beneficial storage environment which could extend storage life and be economic and 2) the changes occurring at this temperature are relatively rapid.

Figure 2. Chinese mustard (Brassica campestris, Chinese group)

MATERIALS AND METHODS

Chinese mustard or Pak Choi (Brassica Campestris, Chinensis group) was used in these experiments. "Slow April", a late maturity cultivar, was grown in Michigan State Research greenhouse from 1987-1990. Plants were grown in 15 cm dia.containers and fertilized with a soluble fertilizer. Uniform plants were harvested 8 weeks after planting.

CA storage set-up: Plants were placed at random in 18.9 L plastic containers and exposed to the following gas mixtures: air (control), 3% oxygen, 5% carbon dioxide in air, and 3% oxygen plus 5% carbon dioxide. The balance of the gas mixtures was nitrogen and gas mixtures were continuously verified by gas chromatography. The flow rate of gases was 100 cc/min in a flow-through system. The temperature during storage was 10°C.

Trim loss: The weight of trimming was the amount cut off due to discoloration or decay on leaves or midribs in order to attain a salable condition.Trim loss was calculated as a percentage of the original weight after 4 weeks of storage.

Chlorophyll measurement: Chlorophyll content of the leaf tissue from second whorl was measured weekly according to the method of Moran(1982). Chlorophyll was extracted from two leaf discs which were punched out by a cork borer (11.5 mm in diameter). These discs were soaked in 5 or 10 ml of N,N-dimethyl formamide for 36 hrs at 5°C and the absorbance of supernatant was determined at 647 and 667 nm. The amount of chlorophylls was calculated as follows: Ctotal= 7.04 A664 + A20.27 A647; Ca= 12.64 A664 - 2.99 A647.

Transmission electron microscopy: Chloroplast ultrastructural changes were

observed after 4 weeks of storage. Sections of leaf tissue from second whorl were cut from similar positions in the leaf as used for chlorophyll analysis. Leaf tissue were cut into 1 mm^3 sections and fixed in 4% glutaraldehyde and 1% osmium tetroxide for 6 hrs at room temperature. Samples were then dehydrated in a graded alcohol series and imbedded in Spurr's resin. Ultrathin sections were cut on a Sorvall MT-2 ultramicrotome using a diamond knife. Sections were stained with urinal acetate and lead citrate and examined using a Phillips 201 electron microscope.

Scanning electron microscopy: Leaf tissues were examined by scanning electron microscopy. Sections of leaf tissue were fixed for 1 hr in 4% glutaraldehyde in 0.1M phosphate buffer at room temperature, then washed three times with 0.1M phosphate buffer, pH 7.2. The samples were dehydrated in a standard ethanol series, freeze fractured in liquid N_2, critical-point-dried (Balzers Critical Point Dryer), and sputter-coated with gold(Emscope Sputter Coater). The samples were viewed in a JEOL JSM-35C Scanning Electron Microscope.

Protein extraction: Five gram of leaf tissue was ground with a pestle in a pre-cooled (4°C) mortar, 25 ml of extraction buffer containing 0.7M sucrose, 0.5M Tris-base, 1.2% polyvinypolypyrrolidone, 0.1M KCl, 0.03M HCl, 0.04M DTT was added and then homogenized until a fine suspension was obtained. The mixture was incubated at 4°C for 10 mins, an equal volume of buffer-saturated phenol was added. This mixture was further shaken at room temperature for 10 mins, centrifuged at 7000 rpm for 10 min, the phenol phase collected. The proteins were precipitated from the phenol phase by addition of 5 volumes of 0.1M NH_4OAC in methanol. These were incubated at -20°C overnight and centrifuged at 7000 rpm for 15 min

and the precipitate washed 3 times with the NH_4OAC in methanol, once with 80% acetone. The pellet was dried under hood. The pellet was then solubilized in adequate volume of extraction buffer.

Protein assay: The protein assay was done according to Bradford (1976) using Biorad (Biorad Laboratories) protein reagent with BSA used as the standard.

Standard Assay Procedure: Several dilutions of protein standard (BSA) were prepared containing from 0.2 to 1.4 mg/ml. A standard curve was prepared each time the assay was performed.

1). 0.1 ml of standard and appropriately diluted samples were placed in clean, dry test tubes and 0.1 ml of the sample buffer was used as the "blank".

2). 5.0 ml of diluted dye reagent was added.

3). The solution was mixed several times by gentle inversion of the test tubes.

4).Over a period of 5 min to 1 hr, The O.D. at 595 nm was measured versus reagent blank.

5).The $O.D._{595}$ versus concentration of standards was plotted and the unknowns read from the standard curve.

SDS PAGE electrophoresis: Polypeptides were analyzed directly by one-dimensional SDS-PAGE electrophoresis. Protein samples were solubilized by boiling for 15 mins in 2% (w/v) SDS, 64mM Tris-HCL (pH 6.8), 5% B-mercaptoethanol, 10% (v/v) glycerol before loading on the gel. The separating gel contained 12.5% acrylamide, 0.33% bisacrylamide, 375mM Tris-HCL (pH8.8) and 0.33% (w/v) TEMED. The stacking gel consisted of 5% acrylamide, 125mM Tris-HCL (pH 6.8), 1% ammonium persulfate and 0.1% TEMED. Each well was loaded with 50 ug of

protein. The running buffer contained 25 mM tris, 192mM glycine and 0.1% SDS. The gels were 1.5mm thick and were run about six hrs at constant current of 30 ma. After electrophoresis, the gels were soaked immediately for about an hour in fixative, then stained in 0.1% Coomassie Brilliant Blue R which was dissolved in 50% (v/v) methanol and 7% (v/v) acetic acid in water to visualize the standards and destained afterwards in stain free solution as above overnight. The migration distances of the calibration proteins, and the proteins of interest were measured, and the corresponding R_f values were determined. The molecular weight of the proteins of interest was determined from its R_f value on the calibration curve.

Ribulose 1,5-bisphosphate carboxylase isolation: Isolation of RUBP-carboxylase was done by the method of Hall and Tolbert (1978) with some modification. All steps were carried out at 4°C. Washed, deveined leaves were ground with mortar and pestle in Bicine buffer(50 mM N,N-bis(2-hydroxyethyl) glycine (Bicine), 1mM $EDTANa_2$ and 10 mM 2-mercaptoethanol,(pH 8.0) with 2% polyvinylpolypyrrolidone (w/v), in the ratio of 2ml of buffer per gram of tissue. The homogenate was filtered through 5 layers of cheese cloth and then miracloth before centrifuging at 23,000xg for 45 mins. The supernatant solution was decanted through 2 layers of miracloth to remove some floating materials. To the supernatant sufficient 60% (w/v) PEG-4000 was added with rapid stirring to make the concentration of PEG-4000 to 18%. After stirring for 30 mins, the solution was centrifuged at 23,000g for 45 mins and the precipitate discarded. A solution of 2M $MgCI_2$ was then added to the clear supernatant to a give final concentration of 20 mM $MgCI_2$. A white protein precipitate containing RUBP carboxylase formed immediately upon addition of

$MgCl_2$. After stirring for 30 mins, the precipitated enzyme was removed by centrifuging at 16,000xg for 30 mins, and redesolved in activating buffer (100mM Bicine, 10mM $NaHCO_3$, 20mM $MgCI_2$, 0.2mM EDTA, 1mM DTT) to make a protein concentration about 2 mg/ml.

Activation and assay of RUBP-carboxylase: The enzyme was activated at a protein concentration of 2 mg/ml at 30°C for 15 mins and diluted 25 fold into the assay mixture in order to minimize the carry-over of activating $NaHCO_3$ in the assay solution. Each 8-ml glass scintillation vial contained 250 ul of assay buffer (200 mM Bicine, 0.4 mM EDTA, 1 mM DTT, pH 8.2), 20 ul of 12.5 mM RUBP (pH6.5), 5 ul of 2 M $MgCI_2$, 20 ul of $NaH^{14}CO_3$ solution (250mM, specific activity >0.2ci/mol) and 150 ul of H_2O.

The reaction was initiated after temperature equilibration by the addition of activated enzyme (i.e. 20 ul). After 30 sec, the reaction was stopped by the addition of 200 ul of 2N HCl. The vials, which contained the acidified samples, were dried overnight under a fume hood to remove excess $^{14}CO_2$ and acid. H_2O (0.5 ml) was added followed by 4.5ml of scintillation cocktail. The radioactivity was counted in LKB 1211 RACKBETA Wallac Liquid Scintillation Counter for 5 mins.

Carboxylase specific activity (umol/min/mg protein)

$$= \frac{^{14}C \text{ (dpm)}}{\text{dpm } (^{14}C/\text{umol}CO_2) * \text{time(min)} * \text{mg protein}}$$

Each data point represents the mean value of triplicate analysis. Analysis of variance was conducted and the means separated using the Duncan's multiple range test when F test was significant.

RESULTS

Mesophyll chloroplasts

It is evident from Figure 3 that the chloroplasts from freshly harvested leaves contained an extensively developed membrane system and they were organized in grana region. The presence of relatively large starch grains in the stroma was a common feature. Several plastoglobuli were observed and they were small.

Figure 4 shows cells from Chinese mustard leaf tissues stored 4 weeks in air (control). Almost all the intercellular contents had disappeared. The cells retained the plasmalemma and cell walls, but the tonoplast has disappeared and the cytoplasm and nucleus are almost completely lost. The chloroplasts are hardly recognizable. Figure 5 illustrates the mesophyll chloroplasts from Chinese mustard leaves stored 4 weeks in 3%O_2. It is evident that the stacking in the grana is less when compared to freshly harvested leaf chloroplasts. There were also numerous large dense plastoglobuli. Some of these appear to protrude into the cytoplasm and vacuole. They lay in the stroma and distorted the thylakoid of the plastids. The amount of stacking in the grana of chloroplasts of leaves stored in 5% CO_2 in air was markedly greater than those from 3% O_2 (Figure 6). The thylakoid system was still extensive; The presence of many grana present could be an indication of photosynthetic activity at this stage. Plastoglobuli were as large but less dense than the 3% O_2 treatment. The plastids from 3% O2 plus 5% CO2 (Figure 7) contained a well developed membrane system, particularly the grana membranes and they had smaller plastobuli. No starch grains were found in any of the chloroplasts from any leaves stored in any treatments after 4 weeks.

Figure 3. Cells from freshly harvested Chinese mustard leaves, showing well developed chloroplasts. A: part of cell; B: whole cell.

CW - Cell wall; CL - Chloroplasts; G - Globules; M - Mitochondria; N - Nucleus; - SG - Starch grains

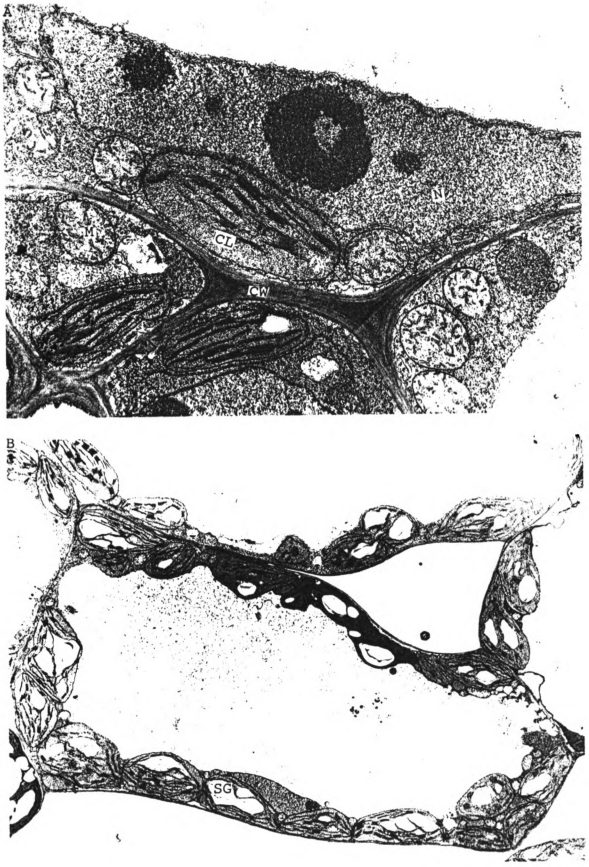
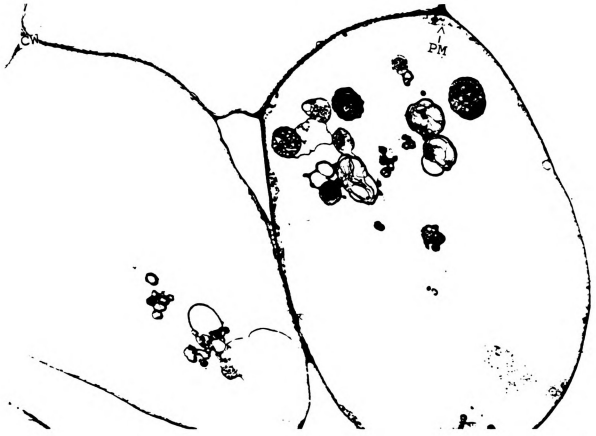

A
N
CL
CW
B
SG

Figure 4. Cells from Chinese mustard leaves after 4 weeks storage in air at 10°C. A: A: whole cell, B: part of cell. CW - Cell wall; PM - plasmalemma



B

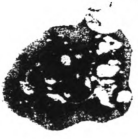
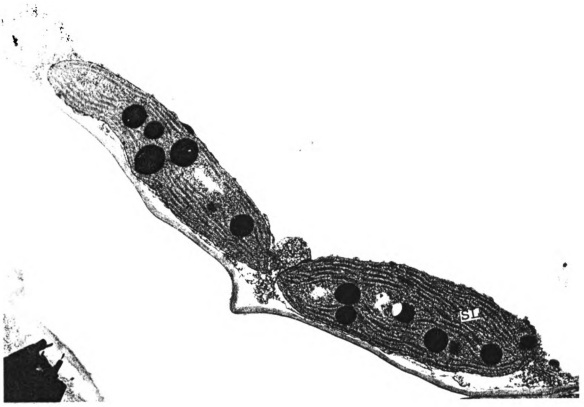


Figure 5. Ultrastructural features of chloroplasts from Chinese mustard leaves after 4 weeks storage in 3%O_2 at 10°C. Note less stacking in grana and learge dense globules.

G - globules; M - Mitochondria; SL - stroma lamellae



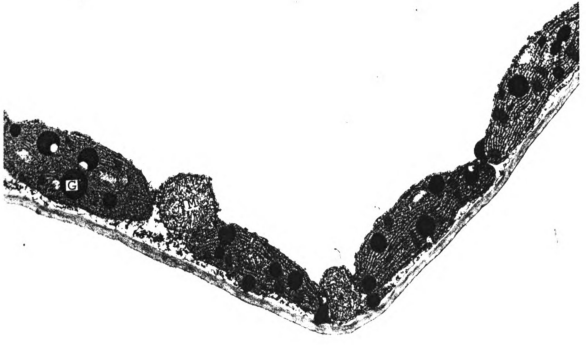


Figure 6. Ultrastructural features of chloroplasts from Chinese mustard leaves after 4 weeks storage in 5%CO_2 and air . Note amount of stacking in grana and large but less dense globules.

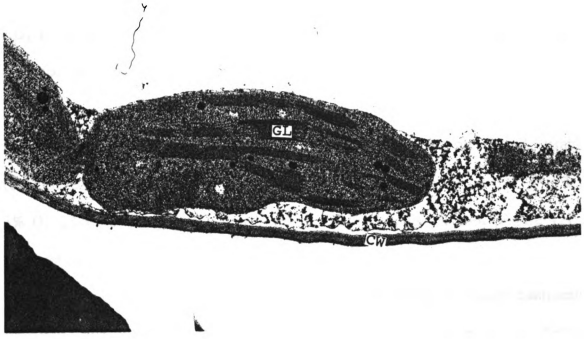
CW - Cell wall; GL - Grana lamellae; G - Globules; M - Mitochondria


GL
M


G

Figure 7. Ultrastructural features of chloroplasts from Chinese mustard leaves after 4 weeks storage in 3%O_2 and 5%CO_2. Note the well developed internal membrane system and small globules.

CW - Cell wall; GL - Grana lamellae; G - Globules


Y
CW


G

Mesophyll cells

In scanning electron micrographs, mesophyll cell were still well-defined (Figure 8, Figure 9 and Figure 10), cell walls maintained intact and rigid from 3%O_2, 5% CO_2 in air and 3% O_2 plus 5% CO_2, whereas cells from air treatment (control) appeared collapsed (Figure 11).

Trim loss

The greatest trim loss after 4 weeks storage was in the air treated sample (controls)(54.1%). CA storage reduced trim loss compared to the control; 44.6% in 3% O_2, 28.8% in 5% CO_2 in air, and 23.3% in 3% O_2 plus 5% CO_2 (Figure 12).

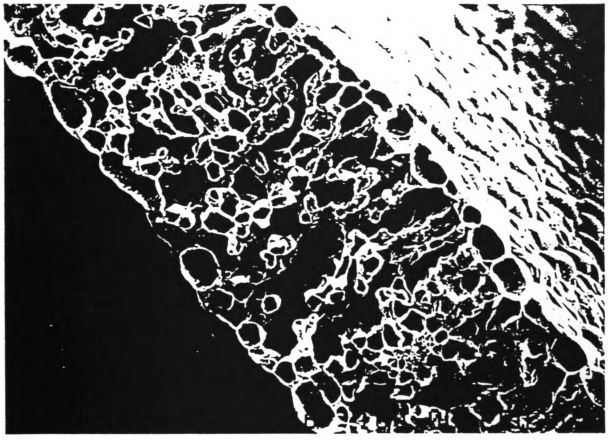
Chlorophyll loss

A continuous loss of chlorophyll occurred in leaves during all storage treatments but more slowly in those stored in CA. Figure 13 and Figure 14 illustrate the changes in chlorophyll a and total chlorophyll over the 4 weeks period. The fastest decline of both chlorophyll a and total chlorophyll was in air. Chlorophylls declined in the 3% O_2 treatment at the same rate as the air treatment up to 2 weeks then declined at a slower rate. Chlorophyll loss in the 3% plus 5% CO_2 and 5% CO_2 in air was the slowest and was significantly different at 1% level by Duncan's multiple range test than the other treatments from the first week.

Soluble protein content

Measurement of protein content of leaf tissues after 4 weeks of storage indicated (Figure 15) that leaf tissues from 5% CO2 plus 3% O2 had the highest protein content (0.44%) in all the the treatments, while leaf tissues from air (control) had the lowest protein level (0.09%). High CO2 (5%) in air maintained higher protein

Fig 8. Mesophyll cell of Chinese mustard leaves after 4 weeks storage in 3%O_2 at 10°C,showing well-defined cells and intact cell walls.



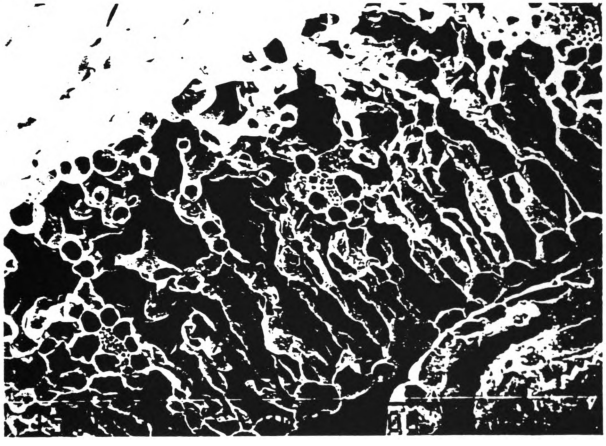
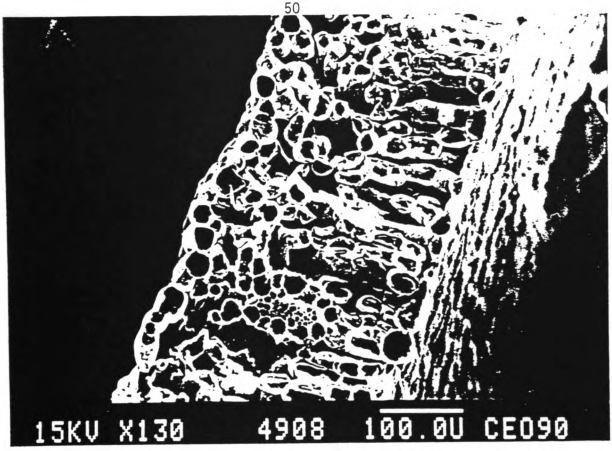


Fig 9. Mesophyll cells of Chinese mustard leaves after 4 weeks storage in 5% CO_2 and air at 10°C, showing well-defined cells and intact cell walls.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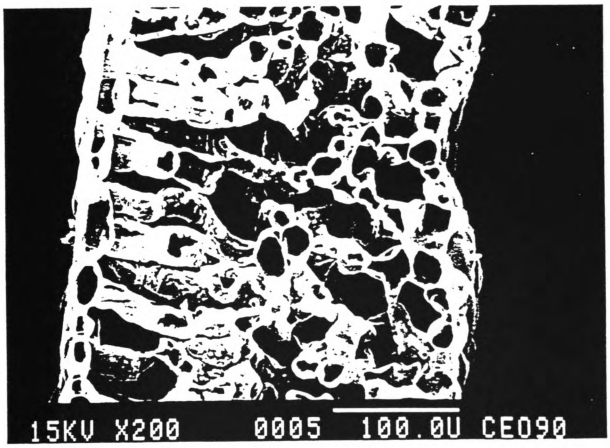
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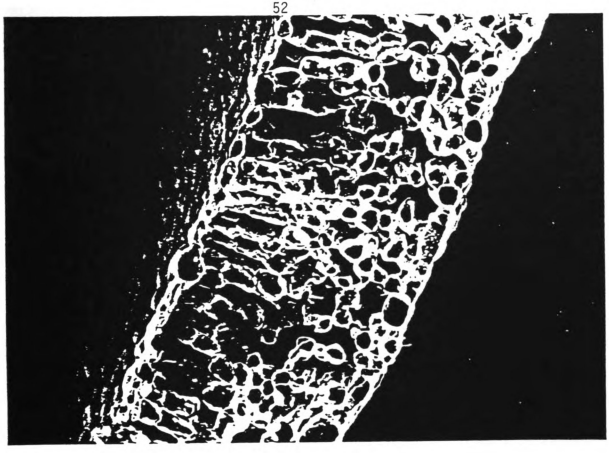
Fig 10. Mesophyll cells of Chinese mustard leaves after 4 weeks storage in 3%O_2 and 5%CO_2 at 10°C, showing well-defined cells and intact cell walls.



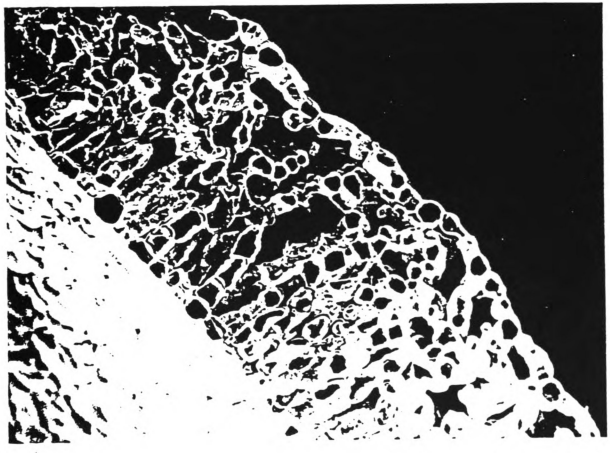
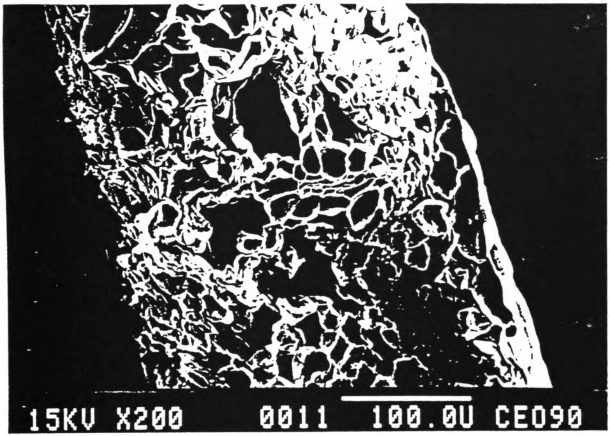


Fig 11. Mesophyll cells of Chinese mustard leaves after 4 weeks storage in air at 10°C, showing collapsed cells.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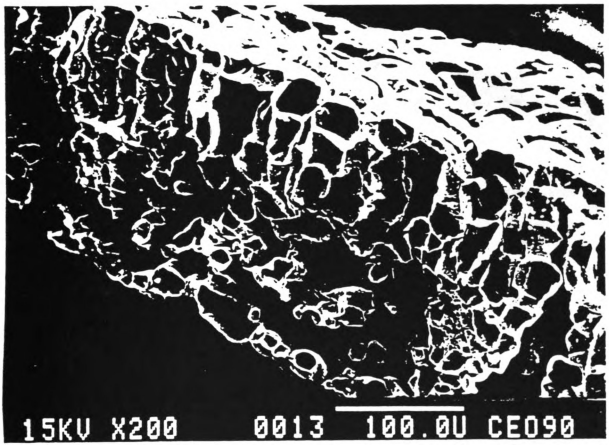
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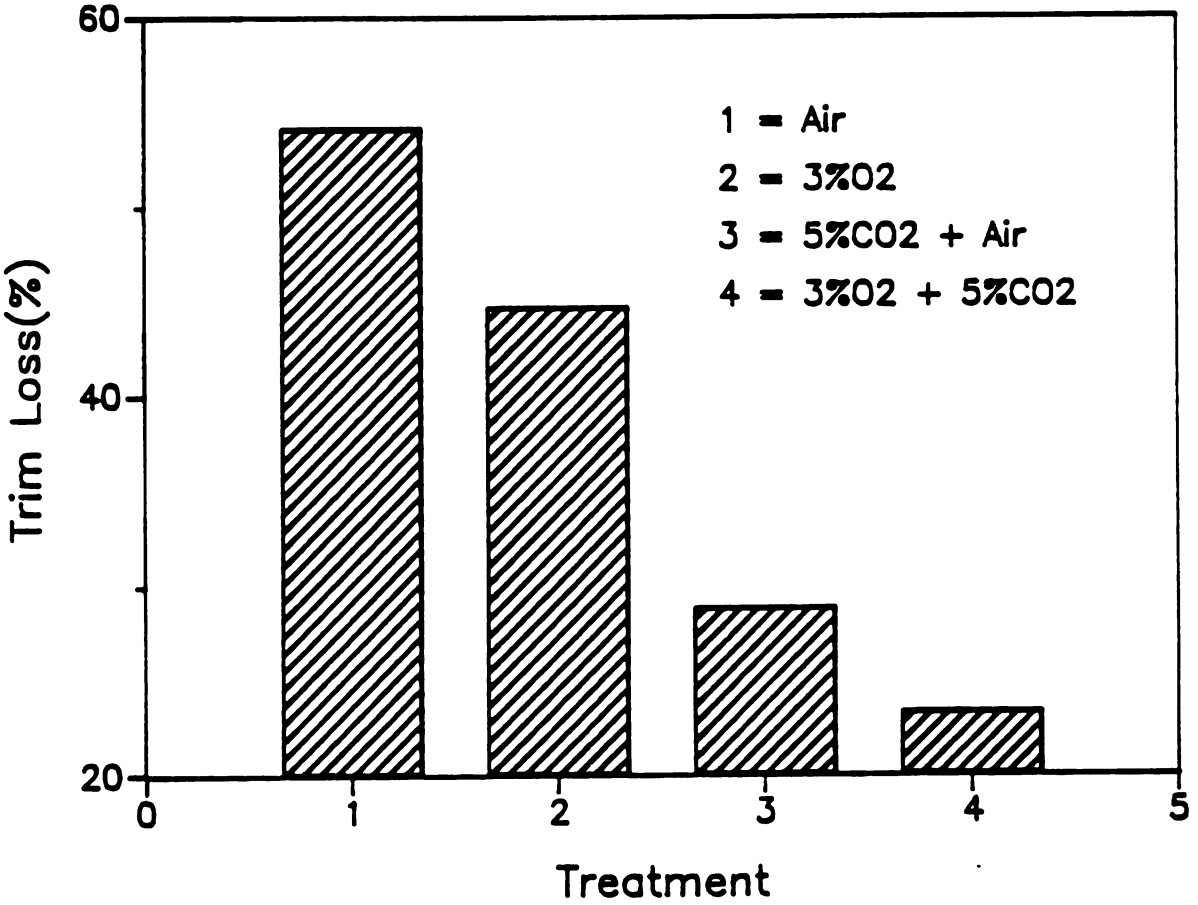

Fig 12. Trim loss of Chinese mustard after 4 weeks CA storage at 10°C. All treatment are significantly different from each other at 1% level by Duncan's multiple range test..

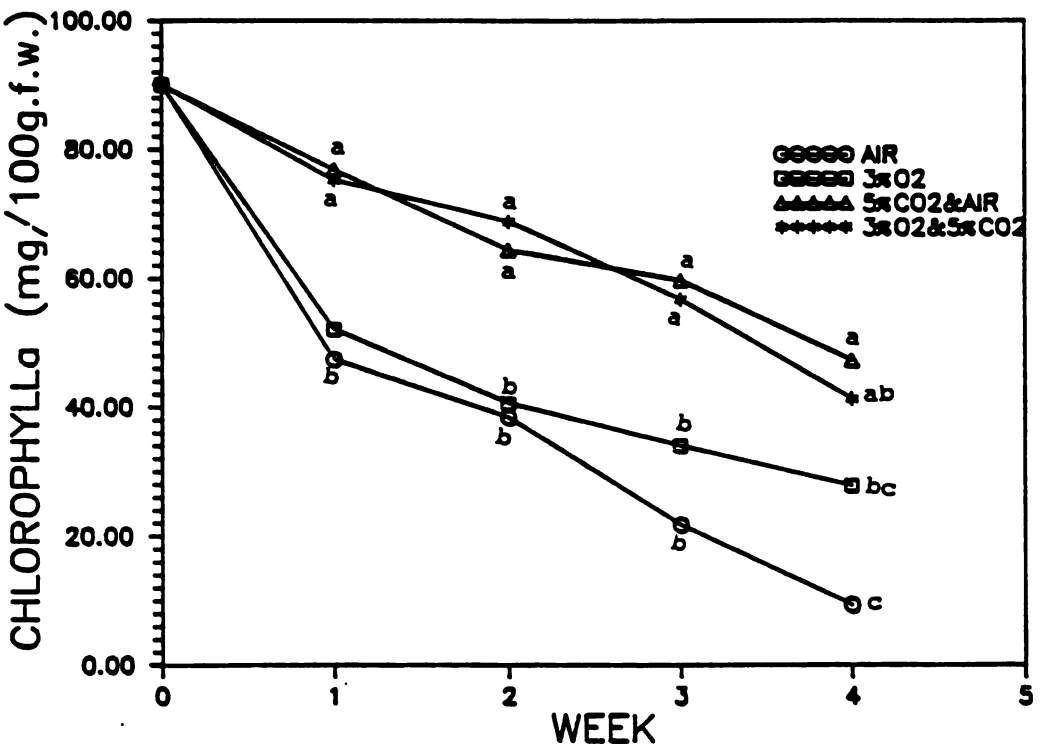

Fig 13. Chlorophyll a loss of Chinese mustard leaves stored in air, 3%O_2, 5%CO_2 in air and 3%O_2 plus 5%CO_2 during 4 weeks at 10°C. Statiatical analysis was conducted by Duncan's multiple range test.

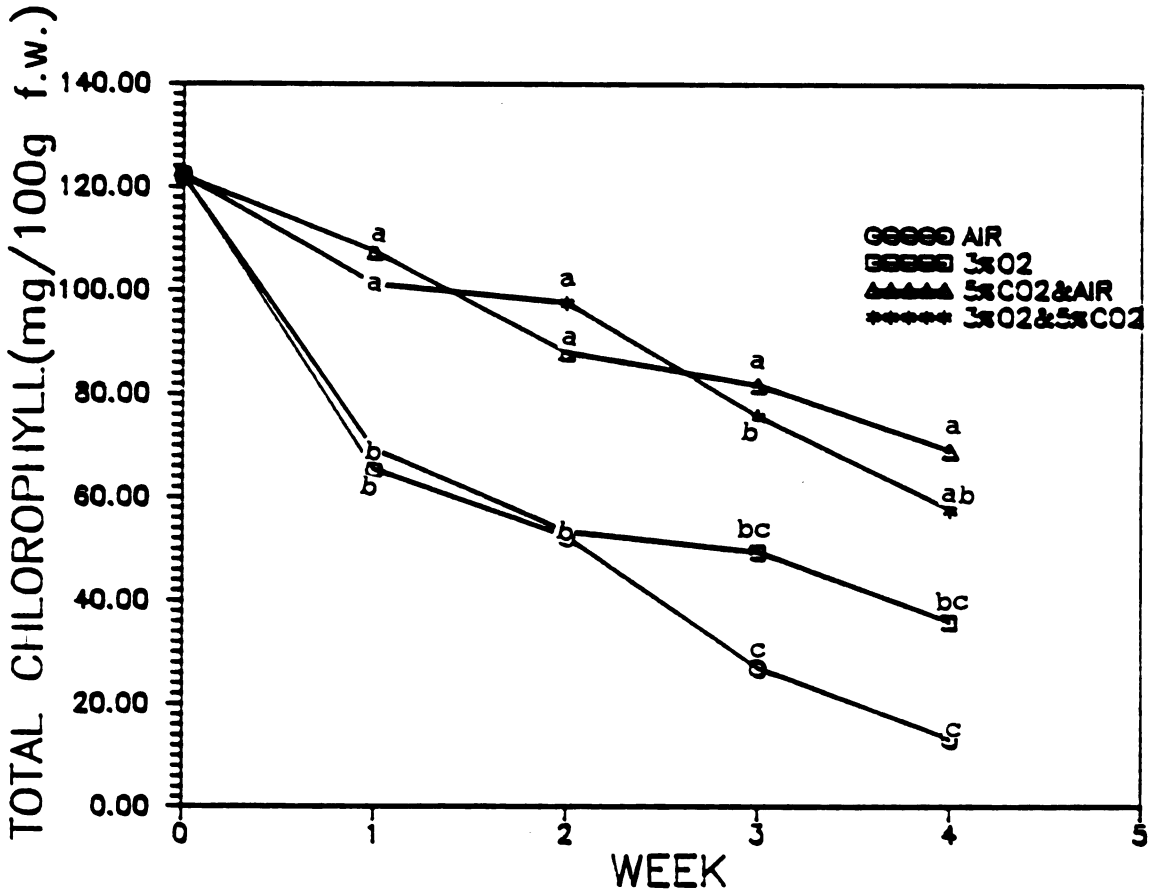

Fig 14. Total chlorophyll loss of Chinese mustard leaves stored in air, 3%O_2, 5%CO_2 in air and 3%O_2 plus 5%CO_2 during 4 weeks at 10^{0}C. Statistical analysis was conducted by Duncan's multiple range test at 1% level.

content (0.32%) than that (0.23%) from low O_2 (3%).

Polypeptide Profile

The pattern of protein of leaf tissues from CA storage were analyzed by SDS-PAGE (Figure 16). A comparison was made among polypeptide profiles to examine the polypeptide composition from CA storage. The protein molecular weight ranged from around 52.5 KD to 15.3 KD. No visual change in protein pattern was observed from CA treatments, but there was a decrease in band size visualized for some polypeptides for example 52.5 (Large Subunit), 48.6, and 15.3 KD(Small Subunit) from the 3% O_2 treatment and these bands were further diminished in the control (air). In the control a loss of bands of 42.7 and 20 KD was observed, and was correlated with, two new bands appeared corresponding to 18.6 and 16.6 KD polypeptides as arrows indicating. Compared to fresh leaf tissue, leaf tissues after CA storage for 4 weeks lost the high molecular weight bands of polypeptides, which were 123, 100, 77.6, 69.2 and 63.1 KD.

Ribulose bisphosphate carboxylase Activity

Similar to protein content, Figure 17 showed that high CO_2 (5%) plus low O_2 (3%) maintained the highest activity of the carboxylase(88.25 nmol/min/mg protein), while leaf tissue from control only had 16.97 nmol/min/mg protein. High CO_2 (5%) in air had higher activity level (74.34 nmol/min/mg protein) than that (58.84 nmol/min/mg protein) from low O_2 (3%).

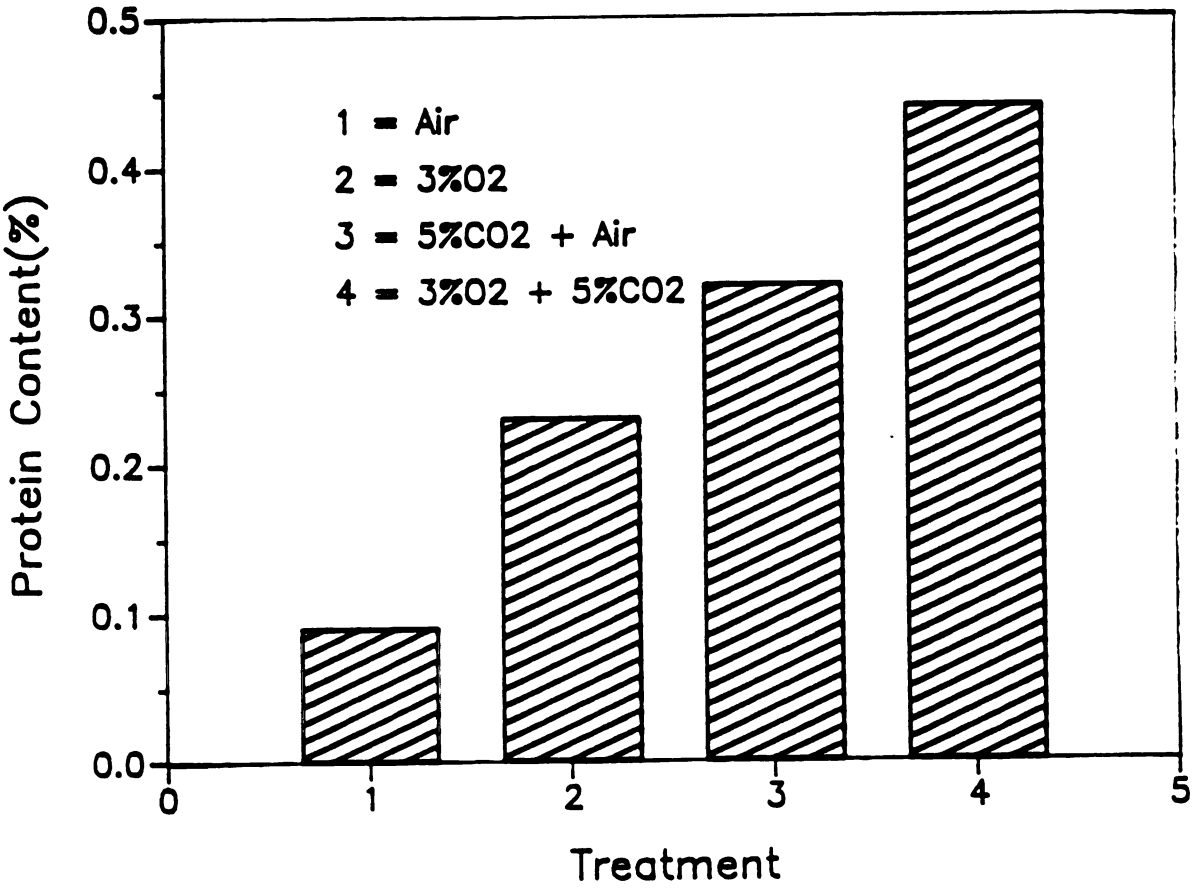

Fig 15. Effect of various CA conditions on protein content of Chinese mustard leaves after 4 weeks of storage at 10°C. All treatnments are significantly different from each other at 1% level by Duncan's multiple range test.

Fig 16. Changes in polypeptide profile of Chinese mustard leaves after 4 weeks storage at various CA conditions at 10°C. Lanes: A, low molecular weight protein markers; B, freshly harvested leaves; C, 3%O2 plus 5%CO2; D, 5%CO2 in Air; E, 3%O2; F, Air (control).


94K
67KD
KD
30KD
20.1KD
A
B
C
D
E
F

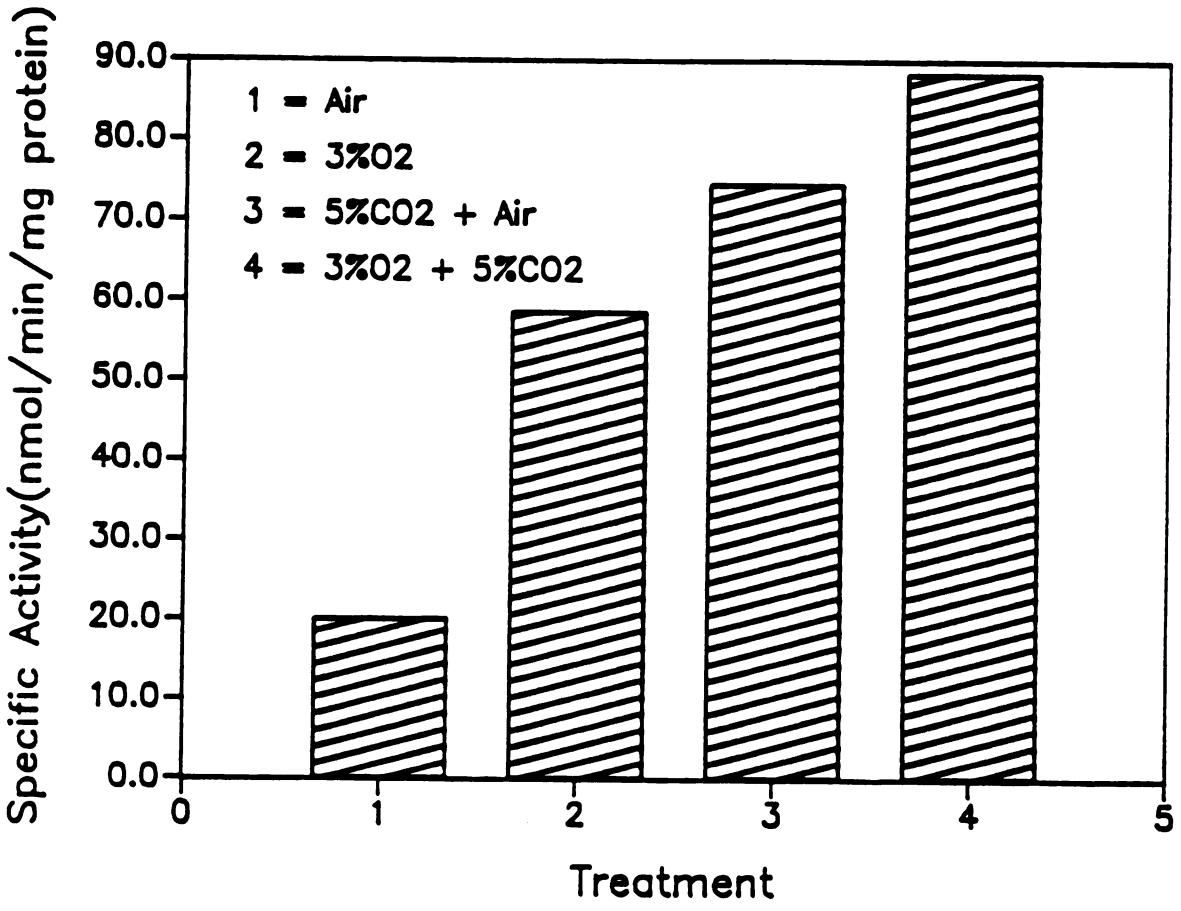

Fig 17. Effect of various CA conditions on ribulose bisphosphate carboxylase activities of Chinese mustard leaves after 4 weeks storage at 10°C. All treatments are significantly different from each other at 1% level by Duncan's multiple range test.

DISCUSSION

CA AND CHLOROPLAST DEGRADATION

As early as 1938, Molish observed, during yellowing of leaves the disappearance of chlorophyll and protein, the disorganization of the chloroplast structure, and the appearance of fat droplets. He found that leaves in a very advanced stage of senescence were unable to fix CO_2 and suggested a correlation between structure and function in the chloroplast. Fine structure changes of senescing leaves have been studied in several plants (Ikeda and Ueda, 1964; Shaw and Manocha, 1965). Senescence of birch tree leaves was associated with a change in shape and volume of chloroplasts in addition to an increase in the number of lipid globules (Dodge, 1970). Since senescence in leaves is tied to chloroplast senescence, knowledge of the mechanism of maintenance of plastids is essential.

The most conspicuous changes in green tissues at the cellular level during senescence is the breakdown of the chloroplasts with its attendant massing of osmiophilic globules. It has been observed in our research that during senescence, chloroplast changes began first; the predominant features were loss of alignment of the grana, loss of compactness in the appearance of the grana and the appearance of electron-dense plastoglobuli.

Freeman et al (1978) found in citrus leaves, that with the conversion of chloroplasts to chromoplasts during senescence, the internal membrane system was reduced and numerous large plastoglobuli appeared. A similar pattern of changes had earlier been reported in discs cut from leaves of Brussels sprouts and floated in the dark (Dennis, et al., 1967). There was first a loosening of the grana which

eventually disappeared to be replaced by a simple lamellar system which aiso later broke down. The chloroplasts became filled with globules. Ikeda and Ueda(1964) have studied the change in structure of chloroplasts in leaf cells of Elodea which were induced to senescence by being floated on water. They observed that during senescence chloroplasts showed a marked decrease in size, the internal thylakoid system was broken down and many large electron-dense globules appeared in the stroma. In control experiments using the light microscope they were able to show that these globules were lipoidal and contained carotenoid pigments, thus causing the well-known yellowing in senescing tissues. Similar changes have been observed in other leaves of cucumber (Butler and Simon, 1971), Nicotiana rustca (Ljubesic,1968) and Phaseolus (Barton,1966).

Small globules are regarded as a normal feature of the chloroplast stroma (Granick, 1961; Menke, 1962;1966). The accumulation of large globules is characteristic of senescing chloroplasts (Butler and Simon, 1971). There is some evidence that the globules are an accumulation of membrane breakdown products as suggested by Ikeda and Ueda (1964). Certainly in some senescing chloroplasts there is an association between the globules and the degeneration of thylakoids (Dennis et al., 1967). The chloroplast globules from a number of plants have been isolated and found to contain mixture of lipids and other substances, not all of which are found in chloroplast membranes. The exact composition of the globules changes with age and it is possible that the globules act as a general store or reservoir of excess lipid and other substances (Lichtenthaler and Peveling, 1967; Lichtenthaler and Weinert, 1970).

This research shows that controlled atmospheres can delay green leaf senescence by maintaining the internal membrane systems (5% CO_2 in air or 3%O_2 plus 5%CO_2) and controlling the enlargement of plastoglobuli (3%O_2 plus 5%CO_2) of chloroplasts. It was found in our experiments that chlorophyll degradation occurred before changes in the internal membrane system of the chloroplasts. In 5%CO_2 in air or 3%O_2 plus 5%CO_2 treatments chlorophyll decreased but there did not appear to be a diminution in the internal membrane system at the same time. This fact also was noticed by Freeman et al. (1978) in citrus leaves. There were not significant differences in number of chloroplast per cell in any of the CA treatments (data not shown).

Compared to chloroplasts from freshly harvested leaves, it was found that the internal membrane system of chloroplasts from leaves treated with 5%CO_2 in air or 3%O_2 plus 5%CO_2 were more developed. It has been suggested by Robertson and Laetsch(1974) that the accumulation of starch may reduce the amount of plastid membrane.

Park and Sane(1971) have reported that PSI and PSII activities are associated with stroma and grana lamellae, respectively. Other workers (Barr et al., 1972) found that large grana stacks were associated with an increase in PSII activities. It is expected that chloroplasts from the 5%CO_2 in air and 3%O_2 plus 5%CO_2 storage treatments would have higher PS II activities than those from the 3%O_2 or air (control) treatment.

Accumulation of osmiophilic globular bodies in senescing plastids indicates a build-up of lipid material originating from thylakoid breakdown (Barton, 1966). The

changing physical state of lipid in chloroplasts of senescing leaves of Phaseolus has been studied by X-ray diffraction (McKersie and Thomson, 1978). It appears that portions of the membrane lipid become more crystalline as senescence proceses. In young leaves the phase transition temperature for thylakoid lipids is below -30°C, but the value changes abruptly during late maturity to +30°C and rises steadily thereafter. The incidence of phase-change in chloroplast lipid at physiological temperatures corresponds to the onset of plastid degradation. Shifts in transition temperature are not attributed solely to alterations in the saturation of the membrane fatty acids, although compositional changes are known to occur (Fong and Heath, 1977). In Phaseolus plastid membrane the dramatic change occurs in the free sterol to phospholipid ratio. Mckersie and Thompson(1978) postulate that this increase in free sterols, together with the loss of chlorophyll and protein helps to bring about a redistribution of polar lipids in the plane of the membrane with the resultant formation of gel state zones. The presence of gel-phase lipid could cause discontinuities in the bilayer at interfaces with other regions still in the liquid-crystalline state and thus contribute to the loss of compartmentation which characterizes senescence.

CA AND CHLOROPHYLL LOSS

Retardation of chlorophyll loss is a major effect of controlled atmospheres. In our experiments, higher carbon dioxide(5%) was more effective than 3% oxygen in this respect. Leberman et al. (1968) stated that an increase in CO_2 is more effective than a reduction in O_2 in slowing color changes; they obtained the same results whether 21 or 3% O_2 was combined with increased CO_2. Wang (1979) tested the influence of

short-term high CO_2 treatments (up to 40%) on the preservation of greenness of broccoli and found that 20% or more CO_2 reduced the decomposition of chlorophyll. Chlorophyll of sweet pepper also is retained better in a CO_2-enriched atmosphere than in air (Wang, 1977; Sarsy,1979). Lyons and Rappaport (1962) reported that a combination of increased CO_2 and decreased O_2 reduced yellowing of Brussels sprouts much more effectively than either alone. Our results supported their findings.

During thylakoid breakdown the two main pigment systems must have different fates. The chlorophyll must be either decomposed or translocated to the cells, whereas the carotenoids, being lipid-soluble, are taken up by the dense globules in senescing plastids. This change in distribution of the pigments in plastids would account for the gradual yellowing of the tissues during senescence.

It was assumed that the effect of low oxygen and high carbon dioxide in reducing chlorophyll loss was related to the inhibition of ethylene production and ethylene action (Lipton and Mackey, 1987). High CO_2 concentration can also increase the pH of cell,Reduced pheophytin formation from chlorophyll at high cell pH is thought to account for chlorophyll preservation. Wang et al. (1971) clearly demonstrated this relation. Pallas(1965) reported that high CO_2 causes stomotal closure and may delay leaf senescence via this route. Our observations under scanning microscope did not find this effect(data not shown).

CA AND PROTEIN DEGRADATION

Loss of total protein is one of the most dominant features in leaf senescence(Martin and Thimann, 1972; Peterson et al., 1973; Wittenbach, 1977). Correlated with this loss is increased proteolytic activity(Feller et al., 1977; Martin

and Thimann 1972; Peterson and Huffaker, 1975). RuBpcase is one of the predominant proteins lost during the initial stages of senescence (Friedrich and Huffaker, 1980; Peterson and Huffaker, 1975). Our results confirmed these observation. Usually (Thomas and Stoddart, 1980), although not always (Pjon, 1981), cycloheximide retards the symptoms of senescence in leaves. Therefore, it is thought that the synthesis of some specific protein in the cytoplasm is required for leaf senescence (Thomas and Stoddart, 1980). In our research, two protein bands with molecular weight of about 18.6 and 16.6 KD, respectively, appeared from more advanced senescing leaves (control). The inhibitor of protein synthesis in chloroplasts, chloramphenicol, retards senescence symptoms in some plants (Sabater and Rodriguez, 1978; Yu and Kao, 1981) but not in others (Thomas, 1975). The indirect approach of the work (Garcia et al., 1983) suggested that, at least in leaf segments, ribosomes with broken rRNA may be active during the earliest stages of senescence. Vonshak and Richmond (1975) found that the activity of protein synthesis of chloroplasts decreased during the senescence of detached tobacco leaves. Changes in the protein synthesized, probably in the cytoplasm, have also been reported during the senescence of wheat leaves (Watanabe and Imaseki, 1982).

The question arises as to what initiates and controls the degradative changes occuring during leaf senescence. It has been shown that a certain proportion of the leaf protein undergoes continuous "turnover", i.e. the protein is being continuously synthesized and broken down, so that the overall rate of change in protein content represents the net differences in the rates of these two processes. Where there is such a continuous turnover, a decline in protein content may reflect a fall in the rate

of synthesis or a rise in the rate of breakdown, or both. The breakdown of protein is brought about by proteolytic enzymes. One possibility that has been suggested by Simon (1967) is that the senescent leaf retains its full capacity for protein synthesis and that the rate of synthesis is limited by lack of amino acids in the leaf. In a healthy, green leaf the amino acids released by protein breakdown are reutilized in further protein synthesis. But it has been suggested that in a senescent leaf amino acids are exported to other parts of the plant so rapidly that there is no "pool" of free amino acids available for protein synthesis, so that there is a decline in protein content. Another postulate of the hypothesis is that the capacity for protein synthesis remains relatively unimpaired during leaf senescence. A convenient method for measuring the rate of protein synthesis is to determine the rate of incorporation of radioactive amino acids, such as ^{14}C-leucine, into protein. Studies of this type (Wareing and Phillips, 1981) have shown that the capacity of tobacco leaves to incorporate ^{14}C-leucine decline during senescence, although quite yellow leaves retain some capacity to synthesize certain enzymes, such as peroxidase and RNAase. It might be argued, however, that the decline in capacity for protein synthesis is the result, rather than the cause of senescence. Overall, it seems that protein metabolism in senescing attached leaves may be viewed as an unbalanced turnover reaction, with catabolism exceeding anabolism. There seems no doubt that the decline in protein content observed in detached leaves arises from a reduced capacity for protein synthesis.

CONCLUSIONS

1. CA storage has effect on reducing trim loss of Chinese mustard. 3%O2 plus 5%CO2 is the most effective treatment which reduced trim loss from 54.1% to 23.3% after 4 weeks storage.

2. 5%CO2 in air is the most effective in decreasing chlorophyll loss of Chinese mustard among the treatments.

3. CA storage can delay chloroplast senescence of Chinese mustard. 3%O2 plus 5%CO2 can maintain the structure of grana and size of plastoglobuli. 5%CO2 in air can keep more stacks of grana than by 3%O2.

4. Cells stored in any of the CA treatments were well-defined and cell walls were rigid. Cells exposed to air were calllapsed.

5. High CO2 (5%) plus low O2 (3%) was the most effective treatment in decreasing the loss of protein from 0.09% (air) to 0.44%.

6. High CO2 (5%) and low O2 (3%) can reduce the loss of RuBPCase activity from 16.97 (air) to 88.25 nmol/min/mg.protein.

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CHAPTER TWO

MODIFIED ATMOSPHERE PACKAGIG OF CHINESE MUSTARD

INTRODUCTION

Several techniques are used to preserve postharvest quality of fruits, vegetables and other perishable produce. These techniques provide optimal temperature, humidity and atmospheric composition. Cooling is probably the oldest and most widely utilized technique, at least in developed countries, to prolong the life of perishable produce. Controlled atmosphere or modified atmosphere storage are other, more sophisticated techniques, that extend the life of certain produce more than does cooling alone, but it is nearly always used in conjunction with refrigeration. The concentrations of CO_2 and O_2 are controlled at the optima specific for each fruit or vegetable. Both of these techniques (refrigeration and CA) are expensive, however, requiring large outlays for installation and maintenance, storage and transport facilities, and they require a great amount of energy, mainly for cooling, but also for establishing and maintaining CA. CA is appropriate for long term storage of large quantities of produce (Ben-Yehoshua, 1985; Kader et al., 1989; Smith et al., 1987). Modified Atmosphere Packaging (MAP) has the potential to provide low O_2, high CO_2 and high RH regimes similar to those of CA storage. Unlike CA storage, such MAP could be applied to shipping containers, retail packages containing several intact or sliced commodity units, or retail packages for individual units of tha commodity (Zagory and Kader, 1988) This technique may extend shelf-life, reduce weight loss and refrigeration costs. It may be better suited

for short term storage of smaller quantities. Significant gains are expected in developing countries. The lack of modern refrigeration and packing house facilities in these countries results in large food losses. The introduction of such a simple technique of preserving perishables might reduce food losses substantially.

MA packages are dynamic systems where respiration and permeation are occurring simultaneously. Factors affecting both respiration and permeation rate must be considered when designing a package. Commodity mass, stage of maturity, temperature, O2 and CO2 partial pressures, ethylene levels and light are known to influence net product respiration in a package. Type, thickness and surface area of the packaging film, as well as temperature, ralative humidity and gradient of CO2 and O2 partial pressures across tha film, are known determinants of permeation. All of the above factors interact to creat equilibrium levels of CO2 and O2 in a sealed package. Package equilibrium is defined as the point when the commodity CO2 production and O2 consumption rates are equal to the permeation rates of the respective gases through a package at a given temperature. A poorly designed package will become anaerobic or develop inacceptable levels of CO2 before equiilibrium is obtained. The ideal package system will equilibrate at the levels of CO2 and O2 that are known to be optimal or enhancement of storage potential of a specific species or variety (Prince, 1989).

Chinese mustard or Pak Choi, an important vegetable in China and Asia, is becoming increasingly popular in areas with large Asian and Pacific island populations in the U.S..Lack of information on postharvest handing and senescence control greatly limits the commercial use of this commodity and contributes to a

large amount of waste. An understanding of the postharvest physiology and senescence of this crop would enable better procedures to be developed to store similar leafy vegetable crops. This would allow supply variation to be reduced and quality to be maintained.

This study was designed to 1) develop a MAP system for Chinese mustard to extend shelf-life and 2) understand how environmental factors affect film permeability, respiratory processes and the interaction of package atmosphere and commodity quality.

MATERIALS AND METHODS

Chinese mustard or Pak Choi (Brassica Campestris, Chinensis group) was used in these experiments. "Slow April" a late maturing cultivar was grown in 15 cm dia. containers in the Michigan State University research greenhouse and fertilized with a soluble fertilizer. Uniform plants were harvested 8 weeks after planting.

Packages were prepared by placing the trimmed plant (one plant/bag) in 1.75, 2.0, 3.0, 4.0 and 6.0 mil thickness, 22 cm X 38 cm surface areas, low density polyethylene (LDPE) (Dow Chemical Co.) bags. The packed bags were then heat sealed. There were two kinds of controls: 1) bags with 8 pin holes, designed for similar RH as heat-sealed treatments but similar gas composition (CO_2 and O_2) as ambient atmosphere; 2) bags with 8 punch holes (dia 7 mm) designed for RH and gas composition similar to ambient atmosphere. Plants packed in 2 mil LDPE bags were held at 0, 5, 10, 15 and 20 °C temperature controlled rooms. Plants packed in 1.75, 3.0, 4.0 and 6.0 mil LDPE bags were held at 10 °C in temperature controlled rooms. Each treatment had three packages as replicate.

One cc gas samples were withdrawn daily for simultaneous O_2 and CO_2 determination to assure the absence of anaerobic conditions. The gas analyzing system consisted of an Ametek Oxygen Analyzer (Model S-3A) and an ADC Infra-red gas Analyzer (Type 225 MK3) connected in series and a strip chart recorder (Linear Instruments Crop.). Nitrogen was used as the carrier gas at 200 ml per minute.

Packed plants were weighed daily to determine weight loss. Weight loss was calculated as follows:

Weight loss (%)=

$$\frac{\text{original weight (g) - weight at sampling time (g)}}{\text{origal weight (g)}} * 100\%$$

Freshness (color and decay) of packed plants was graded by visual examination using "a 9 point scale "method, in which the value 9 was the highest quality as field fresh, value 7 was good , value 5 was fair, value 3 was poor and value 1 was deteriorated.

Trim loss and chlorophyll determination was done as previously described in chapter one. Relative humidity within LDPE packages was measured according to Shirazi and Cameron (1989). A combined temperature and humidity probe (general Eastern, Model 850) was inserted into the bags. Temperature and humidity values were monitored at regular intervals with a datalogger (Omnidata International, Model No. 516B-32).

The permeability of two mil LDPE film to O_2 and CO_2 at various temperatures was determined according to Beaudry (1990). The permeability cell contained two circular 25 ml chambers separated by the film sample, of which 50 cm^2 was exposed to both chambers. Pure O_2 or CO_2 or a mixture of both gases was introduced to one chamber of the cell and N_2 carrier gas was introduced to the other chamber. The rate of O_2 and CO_2 diffusion through the film was calculated from the steady state concentration of the sample gases diffusing through the film and into the carrier gas stream. The concentration of O_2 and CO_2 in the carrier gas stream was determined using the same gas analytical system as above.

The steady state O_2 and CO_2 concentrations in the package and the permeability

data were combined to calculate the rate of respiration using the following formulae:

$$RR_{o2} = P_{o2} * A * X^{-1} * ([O_2]_{atm} - [O_2]_{pkg}) * W^{-1}$$

$$RR_{co2} = P_{co2} * A * X^{-1} * ([CO_2]_{pkg} - [CO_2]_{atm}) * W^{-1}$$

Where RR_{o2} and RR_{co2} are the rates of respiration as measured by O_2 consumption and CO_2 production (m mol/ kg.hr.) respectively; P_{o2} and P_{co2} are measured O_2 and CO_2 permeability coefficients

(n mol.cm./cm^2.hr.KPa), respectively, at the storage temperature; A is film area (cm^2); X is film thickness (cm); $[O_2]_{atm}$ and $[O_2]_{pkg}$ are atmospheric and package concentrations of O_2 respectively; $[CO_2]_{pkg}$ and $[CO_2]_{atm}$ are the package and atmospheric CO_2 concentrations, respectively; and W is plant weight(kg). The RQ was calculated as RR_{co2}/RR_{o2}.

RESULTS

Relative humidity: Table 1 illustrates the RH in LDPE packages of various film thickness at 10 °C. There was no significantly difference in RH between treatments; nor between film thicknesses.

Weight loss: Figure 18 shows the effect of temperature on the weight loss of Chinese mustard packed in 2.0 mil LDPE package. Weight loss of packed plants increased with temperature. Package treatments with pin holes and those sealed decreased weight loss compared with the control with large holes, and there was no difference between packages with pin holes and those sealed (figure 18, 19). No correlation was found between weight loss and film thickness.

Shelf-life: Table 2 showed the effect of temperature on the shelf-life of the Chinese mustard packed in 2.0 mil LDPE packages. Shelf -life of Chinese mustard decreased significantly with increase of temperature. At 10°C and 20°C, treatments had about the same shelf-life.

Table 3 shows the effect of thickness of LDPE package on the shelf-life of Chinese mustard at 10°C. In sealed packages, as the thickness of package increased from 3.0 mil to 6.0 mil, shelf-life of plants increased (figure 20) and was longer than controls (with pin holes) (figure 21, 22,23). Packages of 1.75 and 2.0 mil LDPE had no significant effect on extending shelf-life. Table 4 gives the trim loss and chlorophyll content for plants in 3.0, 4.0 and 6.0 mil LDPE packages after 4 week storage at 10°C.Plants stored in 4.0 and 6.0 mil sealed LDPE packages had less trim loss and higher chlorophyll content.

CO_2 and O_2 concentrations: Figure 24 and Figure 25 shows the changes of CO_2 and

O_2 levels of Chinese mustard packed in various thickness LDPE films during 4 week storage at 10°C. CO_2 and O_2 levels changed dramatically in 1.75, 2.0 and 3.0 mil LDPE package during the first week , then declined until steady state condition reached. In 4.0 and 6.0 mil LDPE packages, CO_2 and O_2 concentrations changed greatly in the first two weeks, then slowed down to steady state conditions. As the film thickness of the package increased, The CO_2 was higher and the O_2 was lower. It should be noted that the change in CO_2 level (from 2.89 % in 1.75 mil to 6.82 % in 6.0 mil) was larger than that of O_2 decrease (from 6.63 % in 1.75 mil to 3.82 % in 6.0 mil).

Steady state CO_2 and O_2 concentrations within 2.0 mil packages at various temperatures are shown in Figure 26. Steady state CO_2 and O_2 levels changed with temperature.

Film permeability: 2 mil LDPE film permeability to O_2 and CO_2 (P_{o2} and P_{co2}) increased with increasing temperature (Figure 27). LDPE has a higher permeability to CO_2 than that to O_2. P_{co2}/P_{o2} increased with temperature.

Respiration and RQ: O_2 consumption and CO_2 production by plants packed in 2 mil LDPE package rose in response to increasing temperature (Figure 28). The RQ was dependent on storage temperature (Figure 29).

DISCUSSION

Storage temperature plays a very important role in determining shelf-life of fresh produce (Prince 1989). Chinese mustard packed in MAP lost more weight as storage temperature increased (Figure 18), which means loss of quality of the fresh produce. Low temperatures extended shelf-life of Chinese mustard (Table 2) since plant tissues had lower respiration rate at lower temperatures (Figure 28). Storage temperature is known to affect the gaseous composition of MAP system for a number of commodities (Beaudry, 1990; Kader, et al., 1989) It is recognized that steady state O_2 and CO_2 level are dependent on film permeability and product respiration and that the temperature dependence of these two processes is determined by film type and commodity physiology, respectively. Our results indicated the respiratory processes of Chinese mustard had greater temperature sensitivities than the respective LDPE gas permeabilities (figure 26, 27 and 28). Beaudry (1990) had the similar results with blueberry fruit. He explained that the interrelationship between temperature, film permeability, respiration and steady state O_2 and CO_2 can be expressed, in general, as follows. If the temperature sensitivity (i.e., the magnitude of changes in response relative to changes in temperature) of the processes of O_2 and CO_2 transmission through the film is less than the temperature sensitivity of the product's respiratory process, then O_2 consumption and CO_2 production will increase more than O_2 and CO_2 permeability as temperature increases. The more rapid increase in respiration leads to a decrease in steady state O_2 . The converse occurs for CO_2, with the steady state CO_2 increasing with increasing temperature.

One of the reasons that MAP has received such attention by the local retail

chains is that it allows them to reduce the fresh produce "shrink" which normally runs at 3-9 % (Lioutas,1988).Our results have comfirmed this.

Atmospheric changes within package plays the leading role in extending shelf-life (Zong, 1989). As the thickness of film increased, steady CO_2 increased and O_2 decreased respectively. CO_2 was 2.36-fold more in 6.0 mil package than that in 1.75 mil package, while O_2 decreased 1.73-fold respectively (Figure 24 and Figure 25). Plants packed in 6.0 mil package had the longest shelf-life and best quality (Table 3, 4 and Figure 20). High CO_2 level may be more effective than low O_2 in delaying leaf senescence of Chinese mustard which was supported by our CA storage experiment. Our preliminary experiment showed that 10% CO_2 could cause injury exhibited as blackness on middle ribs and off-flavour. According to these results the CO_2 level for Chinese mustard should be held between 6-9 %. The specific product has its own particular atmosphere needs and temperature which have been studied and reported by many researchers (Kader, 1980).

MAP has been proved to be an efficient, low cost and energy saving technology, that is suited to the situation in most developing countries which are looking for new efficient ways to reduce the tremendous fresh food spoilage and quality loss.

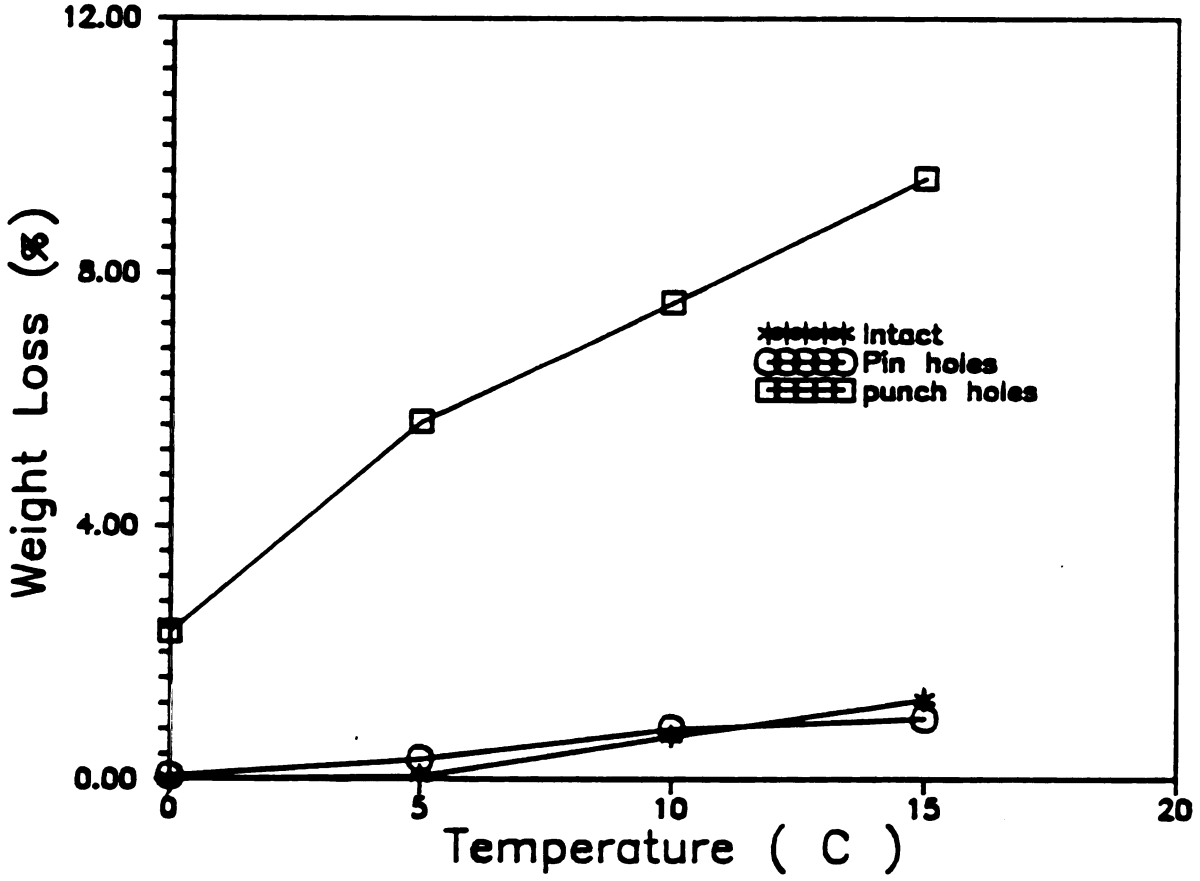

Figure 18. Effects of temperature and package treatment on the weight loss of Chinese mustard in 2.0 mil packages.

Figure 19. Weight loss of Chinese mustard during 4 weeks of storage at 10 °C in packages of various film thickness.

A: intact film packages, B: packages with pin holes, C: packages with punch holes.

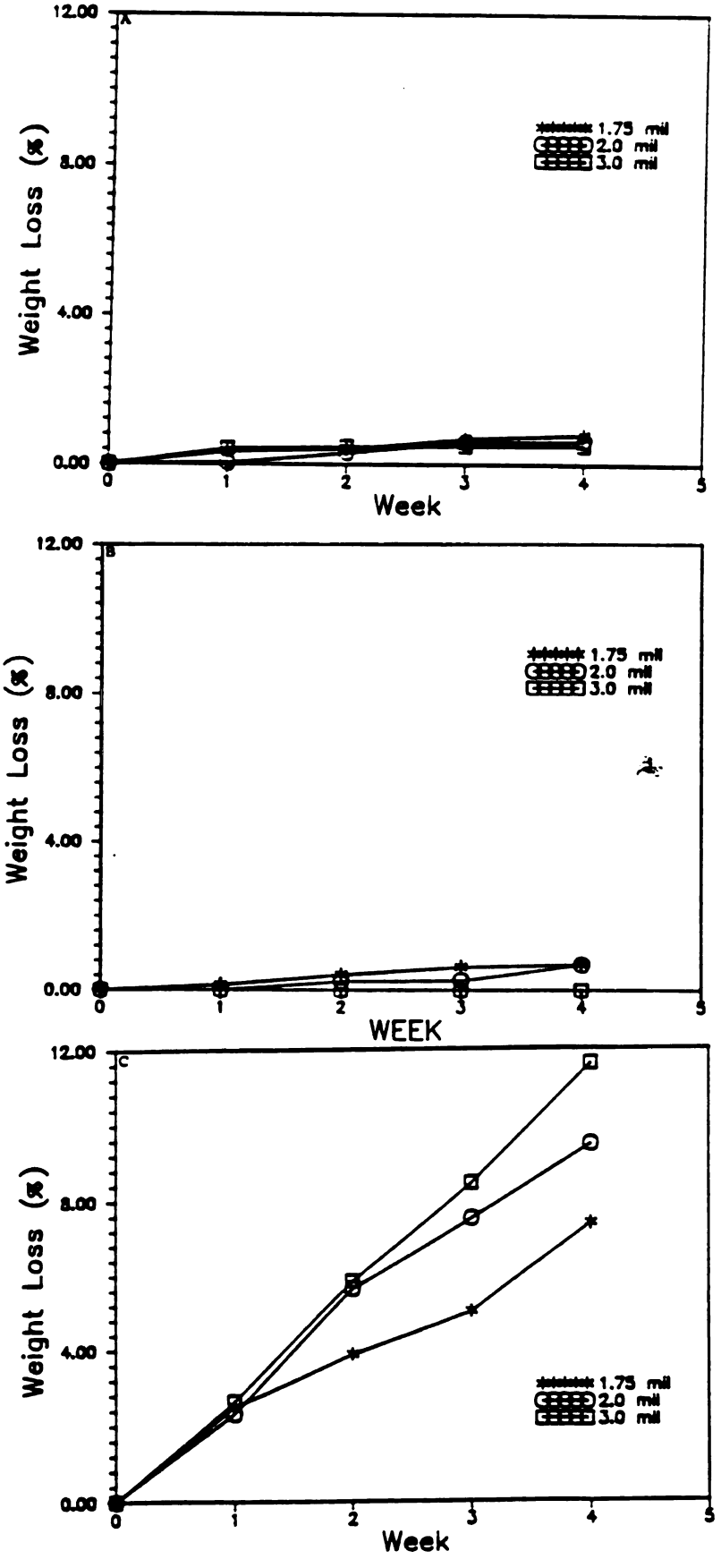
A
12.00
8.00
4.00
0.00
Weight Loss (%)
1.75 mil
2.0 mil
3.0 mil
0
1
2
3
4
5
Week
B
12.00
8.00
4.00
0.00
Weight Loss (%)
1.75 mil
2.0 mil
3.0 mil
0
1
2
3
4
5
WEEK
C
12.00
8.00
4.00
0.00
Weight Loss (%)
1.75 mil
2.0 mil
3.0 mil
0
1
2
3
4
5
Week

Figure 20. Plants of Chinese mustard after 4 weeks of storage at 10 C in packages of different film thickness.


3 MIL (S)
LDPE
4 MIL (S)
LDPE
6 MIL (S)
LDPE

Figure 21. Plants of Chinese mustard after 4 weeks storage at 10°C in 3 mil intact packages (S) or packages with pin holes (F).


3 MIL (S)
LDPE
3 MIL (F)
LDPE

Figure 22. Plants of Chinese mustard after 4 weeks storage at 10 °C in 4 mil intact packages (S) or packages with pin holes (F).


4 MIL (S)
LDPE
4 MIL (F)
LDPE

Figure 23. Plants of Chinese mustard after 4 weeks storage at 10 °C in 6 mil intact packages (S) or packages with pin holes.


6 MIL (S)
LDPE
6 MIL (F)
LDPE

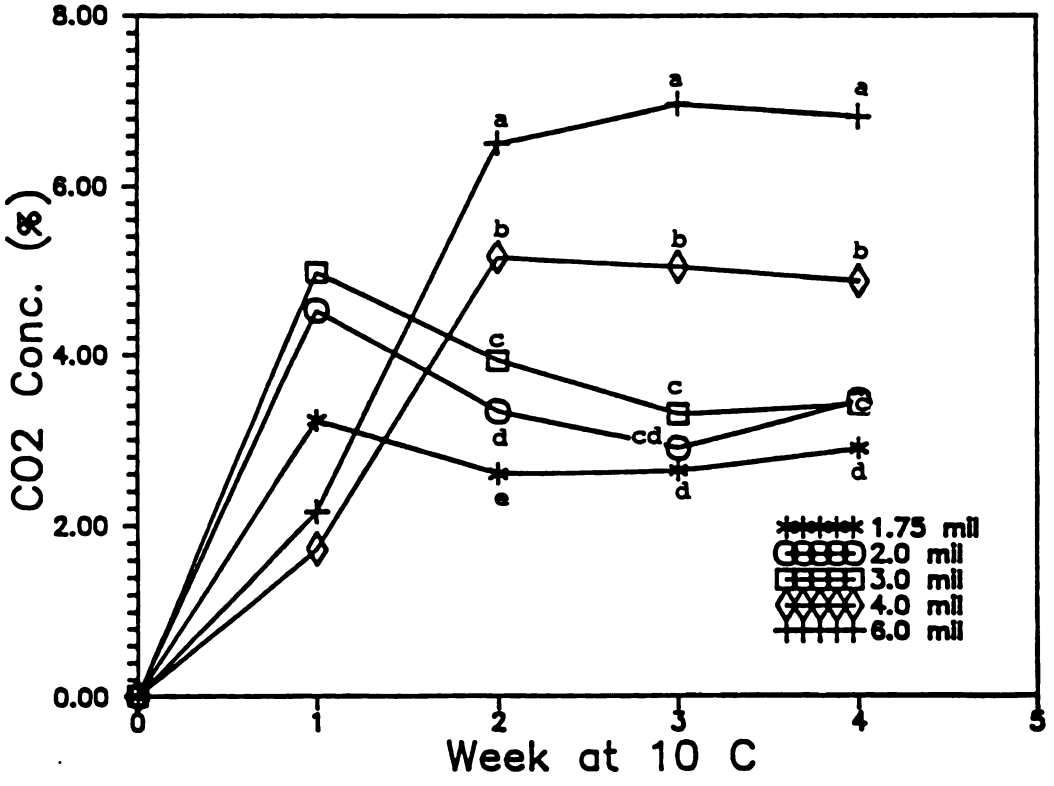

Figure 24. Effect of film thickness on the steady state CO_2 concentrations in packages at 10 °C. Statistical analysis was conducted by Duncan's multiple range test when steady state conditions reached.

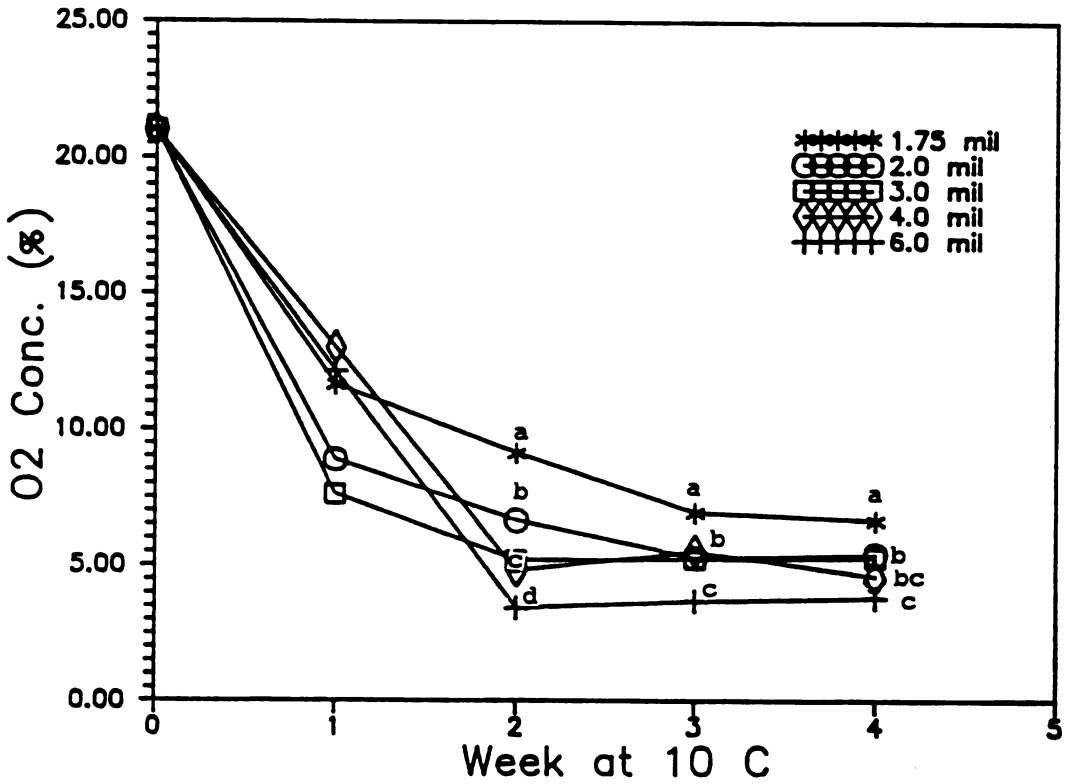

Figure 25. Effect of film thickness on the steady state O_2 concentrations in Packages at 10 °C. Statistical analysis was conducted by Duncan's multiple range test at 1% level when steady state conditions were reached.

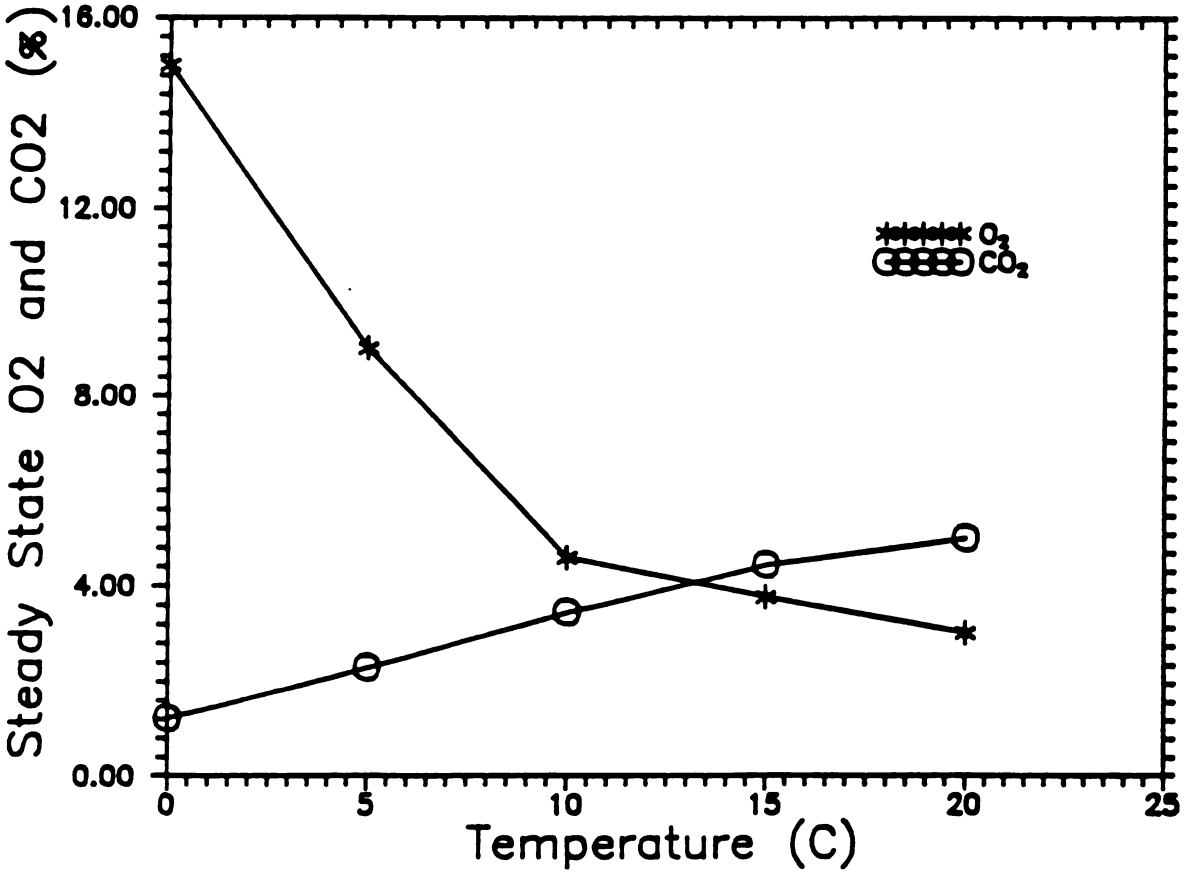

Figure 26. Effect of storage temperature on steady state O_2 level and CO_2 levels in packages packed with Chinese mustard.

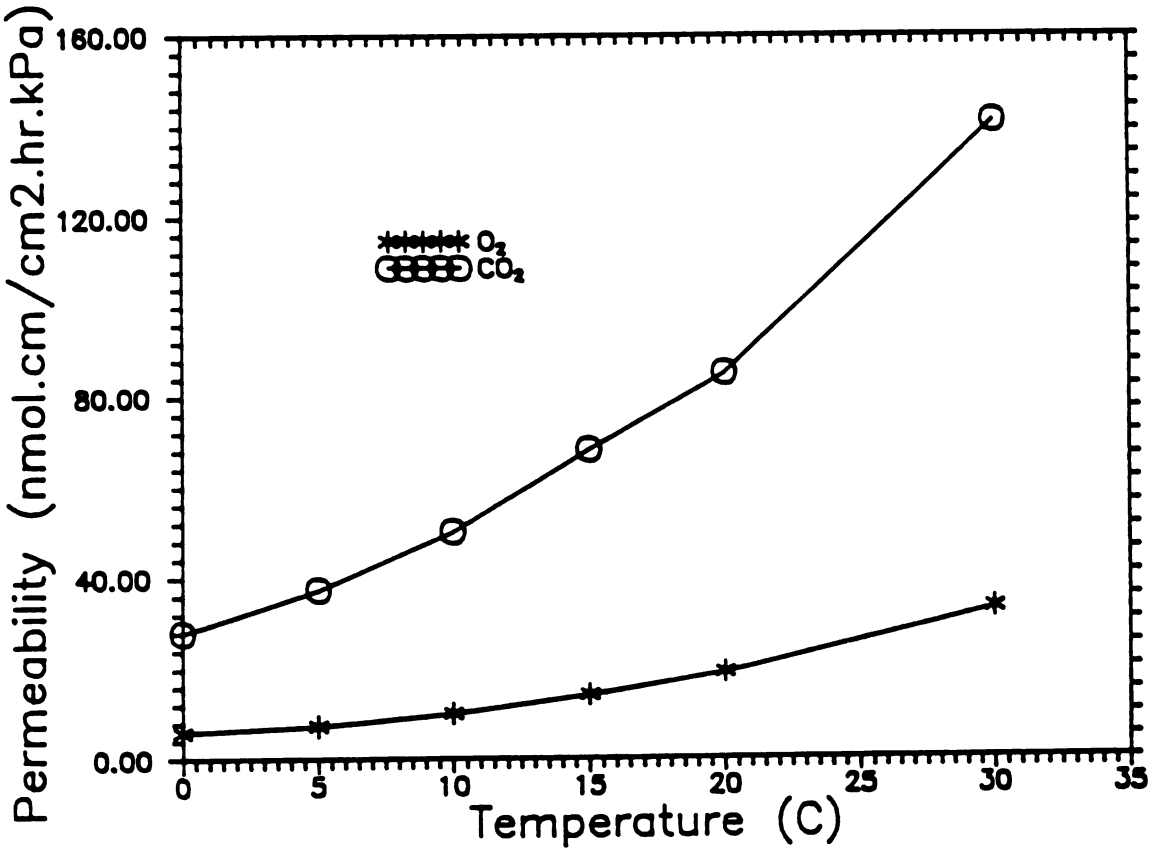

Figure 27. Effect of temperature on P_{o2} and P_{co2} for 2.0 mil LDPE film.

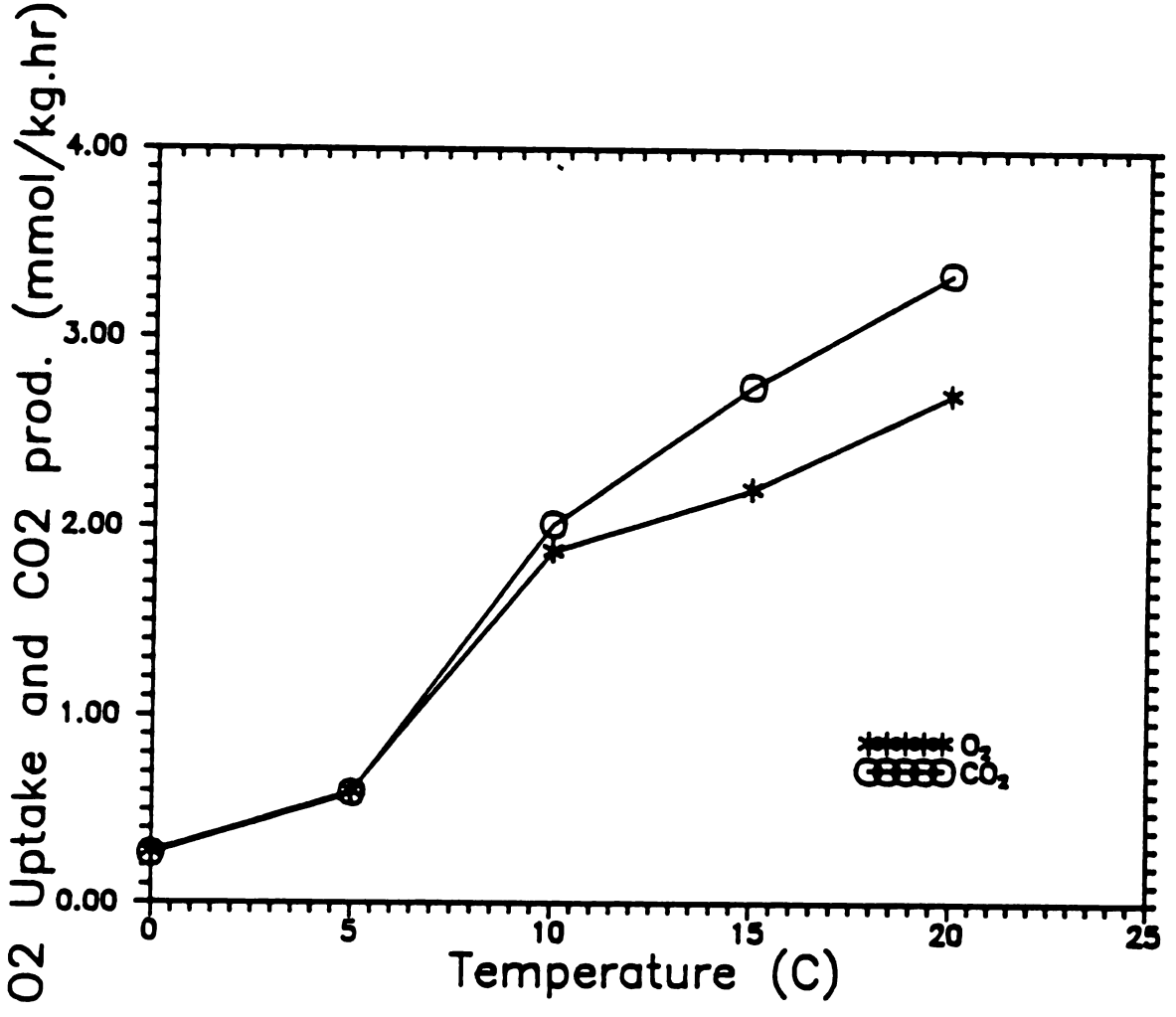

Figure 28. Effect of temperature on O_2 uptake and CO_2 production of Chinese mustard sealed in 22cm X 38 cm, 2.0 mil LDPE packages.

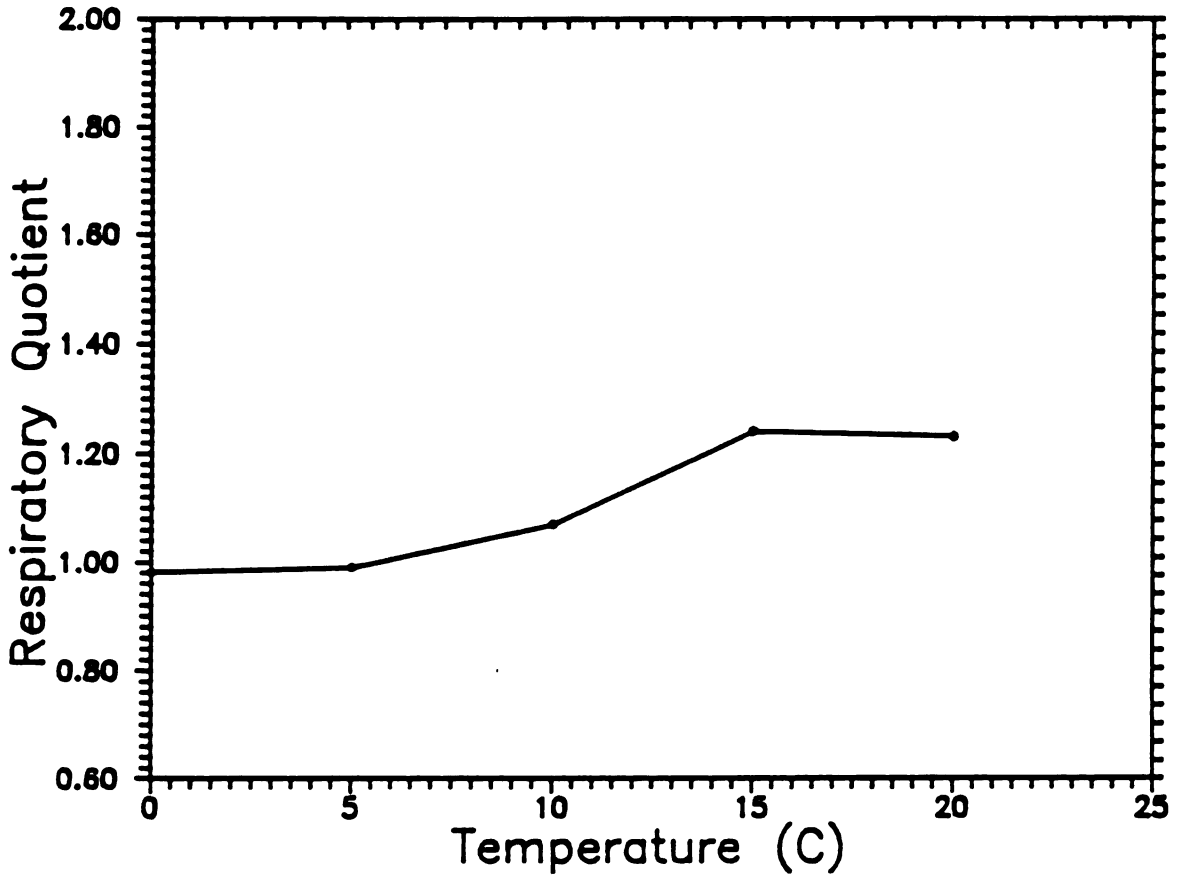

Figure 29. Effect of temperature on respiratory quotient of Chinese mustard sealed 22 cm X 38 cm, packages of 2.0 mil LDPE.

Table 1. Relative humidity of LDPE package packed with Chinese mustard at 10 °C.

Film	Relative humidity (%)*			
Thickness	Bag 1	Bag 2	Bag 3	Room
2.0 mil (none)	95.1	97.0	99.8	81.8
4.0 mil (none)	99.0	98.0	100.8	82.2
6.0 mil (none)	100.0	96.0	100.3	84.9
2.0 mil (pin)	97.0	100.7	99.0	84.9
4.0 mil (pin)	100.0	97.3	99.7	84.9
6.0 mil (pin)	100.0	101.1	98.7	85.3

* not significantly different by LSD.

Table 2. Freshness* of Chinese mustard stored in 2.0 mil LDPE packages at 0, 10 and 20 °C.(9.0- field fresh, 1.0- dead)

Temp.(°C)	Package perforation	Storage duration (weeks) 1	2	3	4
0	none	9.0	9.0	9.0	9.0
	pin holes	9.0	9.0	9.0	9.0
	punch holes	9.0	9.0	9.0	9.0
10	none	9.0	7.7	5.7	3.7
	pin holes	9.0	7.0	7.0	3.7
	punch holes	9.0	6.3	6.3	3.7
20	none	4.3	3.3	1.0	1.0
	pin holes	4.3	1.0	1.0	1.0
	punch holes	7.0	5.7	1.0	1.0

* not significantly different by LSD test.

Table 3. Freshness of Chinese mustard stored at 10°C in 1.75, 2.0 and 3.0 mil LDPE packages (9.0- field fresh, 1- dead).

Film Thickness	Perferation Treatment	Storage duration (weeks) 1	2	3	4
	None	9.0	6.3	5.0	4.3
1.75 mil	pin holes	9.0	6.3	5.7	3.7
	None	9.0	7.7	5.7	3.7
2.0 mil	pin holes	9.0	7.0	7.0	3.7
	None	9.0	7.7	7.0	6.3*
3.0 mil	pin holes	9.0	6.3	6.3	3.7
	None	9.0	9.0*	8.0	7.7*
4.0 mil	pin holes	9.0	6.3	6.3	4.3
	None	9.0	9.0*	9.0*	9.0**
6.0 mil	pin holes	9.0	6.3	6.3	4.3

* means significantly different at 5% level by LSD.

** means significantly different at 1% level by LSD.

Table 4. Trim loss and chlorophyll content of Chinese mustard after 4 weeks of storage in 3.0, 4.0 and 6.0 mil LDPE packages at 10 °C Plants packed in 4 mil LDPE package with pin holes seaved as control.

Treatment	Trim Loss (%)	Chlorophyll (mg/100 g F.W.)
Control	60.5	22.47
3.0 mil	53.4	27.53
4.0 mil	34.9*	44.40
6.0 mil	14.4**	111.93**

* Means significantly different at 5% level by LSD.

** means significantly different at 1% level by LSD.

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