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Effects of Low-oxygen Atmosphere on Ethanolic Fermentation in Fresh-cut Carrots

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Abstract. **Carrot (*Daucus carota* L.) root shreds were stored under a continuous flow of 0.5% and 2% O_2 (balance N_2) or in air for 7 days at 5 and 15 °C to study the regulation of ethanolic fermentation metabolism. Low-O_2 atmospheres of 0.5% and 2% caused increases in ethanol and acetaldehyde concentrations and the activities of alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) compared to air. By day 3, ethanol increased 38-, 25-, 13- ,and 9.5-fold; acetaldehyde increased 20-, 13-, 7.7-, and 5.6-fold; ADH increased 7.6-, 6.3-, 3.8-, and 2.7-fold; and PDC increased 4.2-, 3.9-, 2.3-, and 2.2-fold in samples at 0.5% O_2 at 15 or 5 °C and at 2% O_2 at 15 or 5 °C, respectively, compared with corresponding samples in air. These results indicate that ethanolic fermentation was accelerated more in the 0.5% than in the 2% O_2 atmosphere and more at 15 °C than at 5 °C. The acceleration of ethanolic fermentation may allow production of some ATP, which may permit the carrot tissues to survive.**

Although a number of studies have been conducted to evaluate the effects of controlled and modified atmosphere on the quality and storability of carrot roots (Abdel-Rahman and Isenberg, 1974; Carlin et al., 1990; Hansen and Rumpf, 1974; Leshuk and Saltveit, 1990; Liew and Prange, 1994; Phan et al., 1973; Van den Berg and Lentz 1966; Wills et al., 1979), little is known about the mode of action of low O_2 and high CO_2 on respiratory metabolism of carrots. In a study on the effects of low-O_2 atmospheres on glycolysis of carrots, we reported that glycolytic flux increased under low O_2 due to increased activity of pyrophosphate-dependent : phosphofructokinase, which was activated by the increase in fructose 2,6-bisphosphate (Kato-Noguchi and Watada, 1996a, 1996b). Since hypoxic condition increased ethanolic fermentation in many plants (Davies, 1980; Ke et al., 1995; Kennedy et al., 1992), low-O_2 atmosphere may increase the metabolism of ethanolic fermentation in carrots. Leshuk and Saltveit (1991) reported on the ethanol concentration in carrot root disks 4 h after applying a low-O_2 atmosphere; however, to our knowledge, there is no information on long-term effects of low-O_2 atmosphere on fermentative metabolites and related enzymes in carrots.

Our objective was to investigate the effects of low O_2 on ethanolic fermentation of fresh-cut carrot roots. Levels of acetaldehyde and ethanol and activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were determined in fresh-cut carrots stored under different O_2 atmospheres and temperatures.

Materials and Methods

Plant materials and treatments. Carrot roots were purchased from a local wholesaler, shredded (≈50 mm long, 5 mm wide, and 4 mm thick), and stored in 3.8-L glass jars (100 g fresh mass per jar) at 5 and 15 °C as previously described (Kato-Noguchi and Watada, 1996a). Jars were initially flushed with air for 2 h to dissipate respiratory and physiological responses of wounding (Leshuk and Saltveit, 1991). Then, a stream of 0.5% or 2% O_2 (balance N_2) or air was metered through the jar at 10 mL·min^{-1} at 5 °C and 15 mL·min^{-1} at 15 °C, which was sufficient to keep CO_2 accumulation <0.3%. In the preliminary study, no off-flavor or taste was noted with carrot shreds held in these atmospheres for 7 d.

For determining enzyme activity, three jars of each treatment were removed daily during storage, and carrot shreds were frozen immediately in liquid N_2 and stored at –80 °C until analysis. Samples of day 0 were collected after ventilation with air.

Extraction and determination of ethanol and acetaldehyde. The carrot shreds (20 g fresh mass equivalent) were immediately homogenized with 50 mL of ice-cold 0.1 M HCl after sampling using a Waring blender according to Ke et al. (1995). The homogenate was filtered through four layers of cheesecloth, and a 5-mL aliquot of extract was transferred to a 10-mL screw-cap test tube that was sealed with a Teflon septum.

After incubation for 20 min at 70 °C, a 1-mL sample of headspace gas was injected into a gas chromatograph (GC-12A; Shimadzu, Kyoto, Japan) equipped with FID detector (at 160 °C) and a Porapac Q column (3 mm × 3 m). The flow rate of carrier gas (N_2) was 20 mL·min^{-1}, and the oven was kept at 140 °C. The ethanol and acetaldehyde concentration in the sample was calculated from the peak area. Internal standards for ethanol and acetaldehyde, added to the extraction medium before sample homogenation, showed 80% ± 5% and 82% ±7% recovery, respectively, as calculated from five replications.

Measurement of pH. Carrot shreds (20 g fresh mass equivalent) were homogenized in Waring blender, and the pH of the homogenate was immediately measured with pH meter (601A, Orion, Cambridge, Mass.) (Leshuk and Saltveit, 1991).

Extraction and assay of enzymes. Frozen carrot shreds (2.5 g fresh mass equivalent) were ground to a fine powder in liquid N_2

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using a mortar and a pestle. Before the liquid N_2 had evaporated, 10 mL of ice-cold 100 mM MES buffer (pH 6.5) containing 2 mM dithiothretol and 2.5 g insoluble PVP was added to the sample and the mixture was homogenized. The homogenate was filtered through four layers of cheesecloth and centrifuged at 30,000× *g* for 25 min at 4 °C. The resulting supernatant was used immediately for measuring ADH and PDC activities.

Quantification of enzyme activity was performed spectrophotometrically by monitoring the oxidation of NADH at 340 nm for 15 min at 30 °C in the following 1-mL reaction mixture according to Nanos et al. (1992): 85 mM MES (pH 6.5), 0.9 mM NADH, 0.02 mL sample, and 5 mM acetaldehyde to initiate the reaction for ADH; 85 mM MES (pH 6.5), 0.5 mM thiamine pyrophosphate, 5 mM $MgCl_2$, 0.9 mM NADH, 14 units of ADH, 0.2 mL sample, and 5 mM Na-pyruvate to initiate the reaction for PDC. The overall recovery of enzyme activity by this method was 91% ±4% and 87% ± 5% for ADH and PDC, respectively, according to five replicate assays with the pure enzymes. Protein was determined according to Bradford (1976) using bovine gamma globulin as a standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS. Nondenaturing electrophoresis was performed using slab gels (1.5 mm thick) as follows. Frozen carrot shreds were extracted, and the extract were clarified by centrifugation as previously described. Each gel track contained 85 µL of sample plus 15 µL of 90% glycerol containing 0.01% bromophenol blue. The running gel containing 8.8% (w/v)

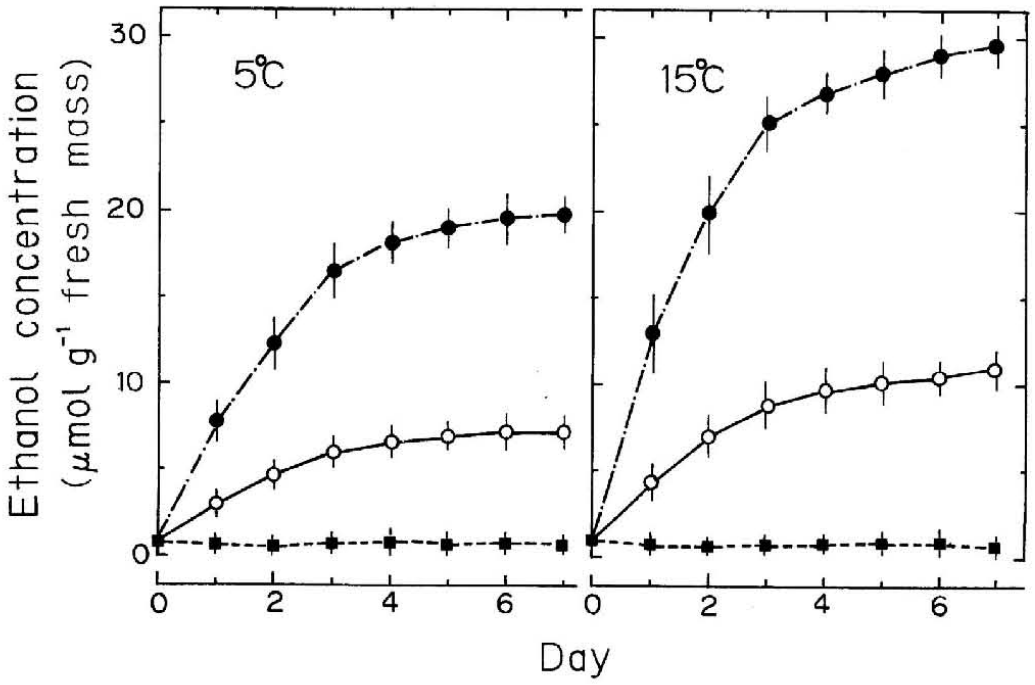

Fig. 1. Changes in the concentration of ethanol in carrot shreds held in 0.5% (●) or 2% O_2 (○) atmospheres and air (■) at 5 and 15 °C. Data are means ± SE from three experiments with three assays for each determination.

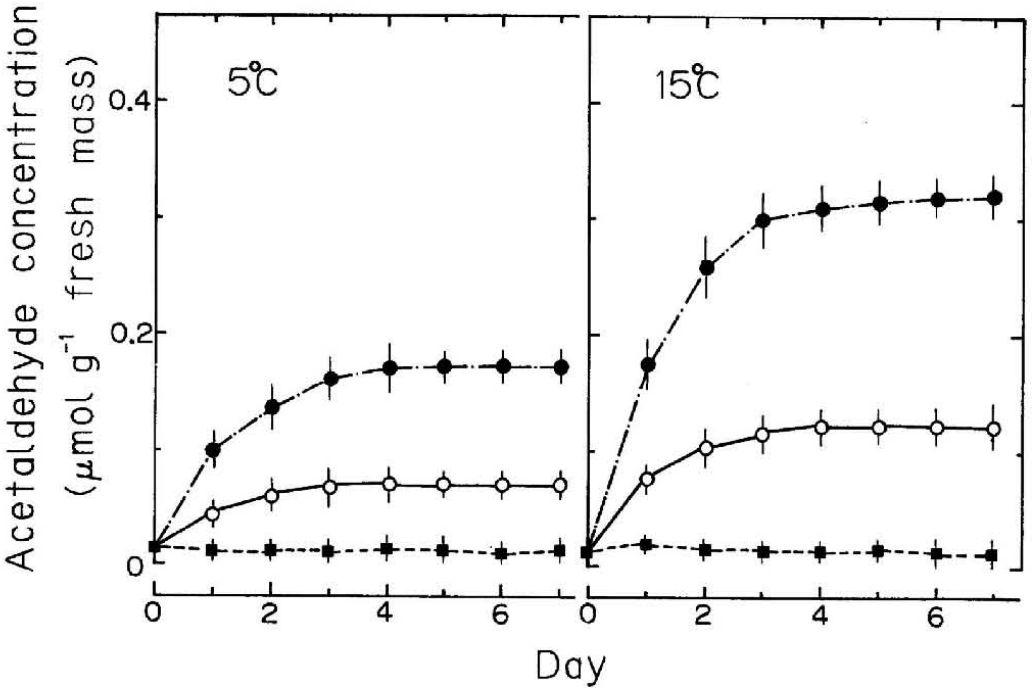

Fig. 2. Changes in the concentration of acetaldehyde in carrot shreds held in 0.5% O_2 (●), 2% O_2 (○) atmospheres and air (■) at 5 and 15 °C. Data are means ± SE from three experiments with three assays for each determination.

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acrylamide-0.12% bisacrylamide, 0.38 M Tris-HCl (pH 8.8), and 12% (w/v) glycerol was polymerized with 0.08% (v/v) TEMED and 0.036% ammonium persulfate. The stacking gel containing 5% acrylamide-0.07% bisacrylamide, 0.075 M Tris-HCl (pH 6.8), and 12% glycerol was polymerized with 0.06% (v/v) TEMED and 0.005% ammonium persulfate for ≈5 h. Gels were run with 10 mM Tris-77 mM glycine buffer (pH 8.8) in both electrode tanks at 15 mamps for 20 h at 4 °C and were stained in a solution containing 100 mL of 0.1 M Tris-HCl (pH 8.0), 50 mg NAD, 5 mg phenazine methosulfate, 15 mg MTT, and 3 mL absolute ethanol (Arulsekar and Parfitt, 1986). ADH bands appeared within 20 min, and development was allowed to proceed an additional 30 min before the reaction was stopped by rinsing in water

Results and Discussion

Concentration of ethanol in the carrot shreds was low on day 0 and did not change during storage in air at either storage temperature (Fig. 1). Ethanol in carrots under low-O_2 atmospheres accumulated rapidly during the first 3 d and only slightly thereafter. Accumulation of ethanol was much greater at 0.5% than at 2% O_2 and at 15 than 5 °C. On day 0, the concentration was 0.57 and 0.61 $\mu mol \cdot g^{-1}$ (fresh mass basis) for samples at 5 and 15 °C, respectively. By day 3, the ethanol increased 38- and 13-fold with samples in 0.5% and 2% O_2 at 15 °C, and 25- and 9.5-fold with samples in 0.5% and 2% O_2 at 5 °C, respectively, compared with samples in air. The greater accumulation at 15 °C was probably due

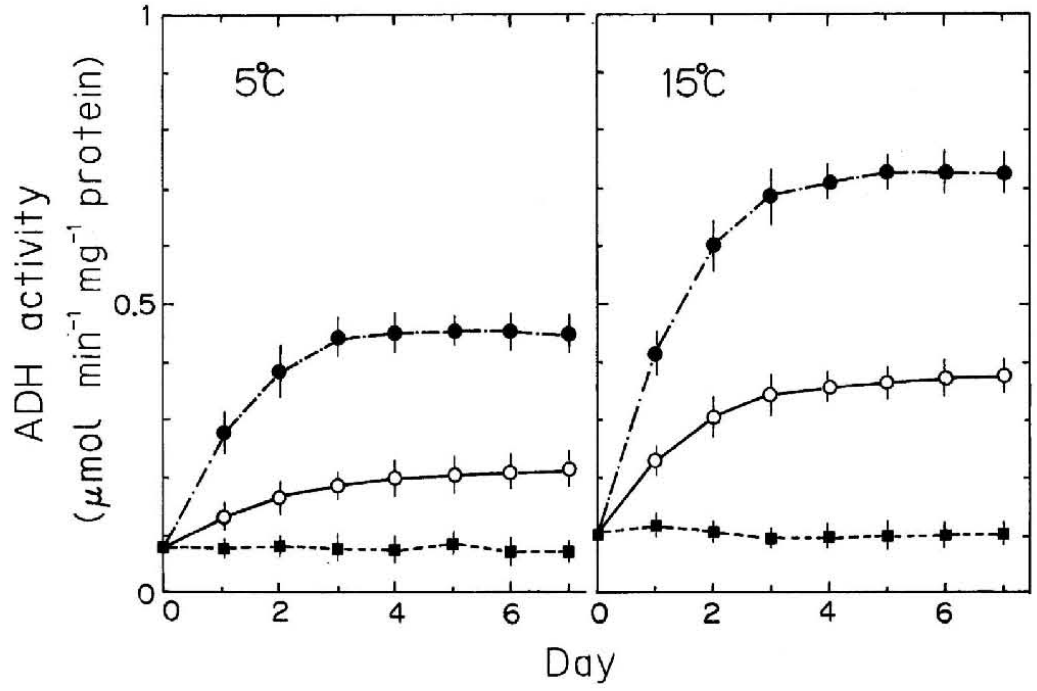

Fig. 3. Changes in the level of ADH activity in carrot shreds held in 0.5% (●) or 2% O_2 (○) atmosphere, and air ((■) at 5 and 15 °C. Data are means ± SE from three experiments with three assays for each determination.

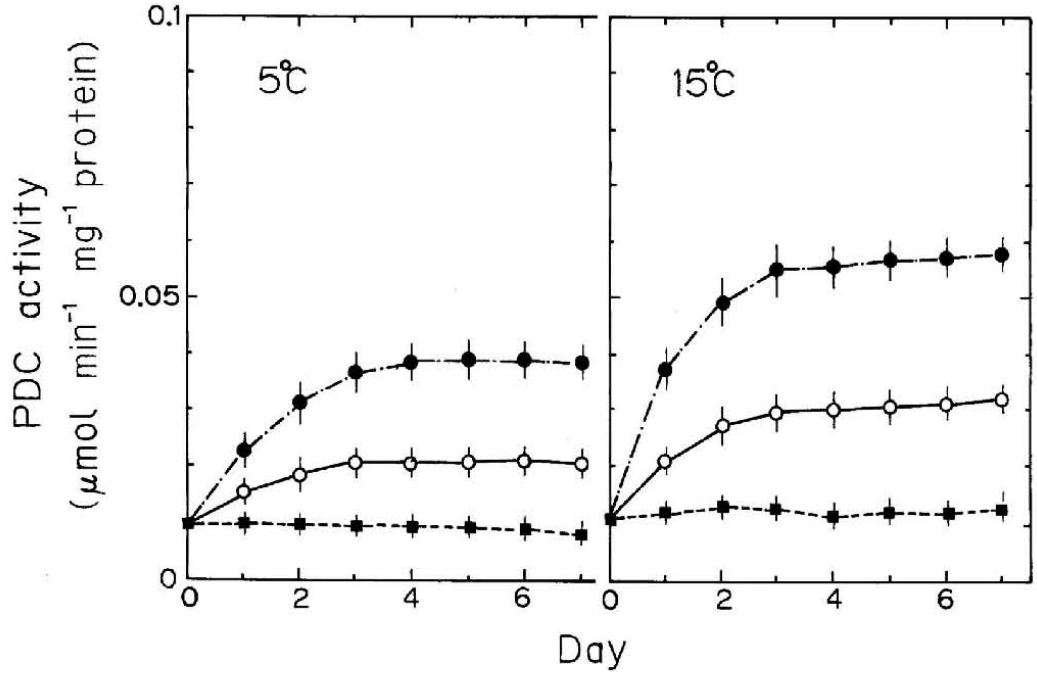

Fig. 4. Changes in the level of PDC activity in carrot shreds held in 0.5% (●) or 2% O_2 (○) atmosphere, and air (■) at 5 and 15 °C. Data are means ± SE from three experiments with three assays for each determination.

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to the higher metabolism rate at 15 °C than at 5 °C. The average accumulation rate of the ethanol was 8.0, 2.6, 5.2, and 1.9 $\mu mol \cdot g^{-1} \cdot d^{-1}$ (fresh mass basis) by day 3 and 1.6, 0.8, 0.9, and 0.3 $\mu mol \cdot g^{-1} \cdot d^{-1}$ (fresh mass basis) after day 3 for samples in 0.5% and 2% O_2 at 15 and 5 °C, respectively. This decreasing accumulation after day 3 may be due to diffusion of ethanol out of carrot tissues or metabolism of ethanol to other compounds (Ke et al., 1995). Leshuk and Saltveit (1991) determined the lag time before increase of ethanol in carrot disks under anoxia to be ≈30 min.

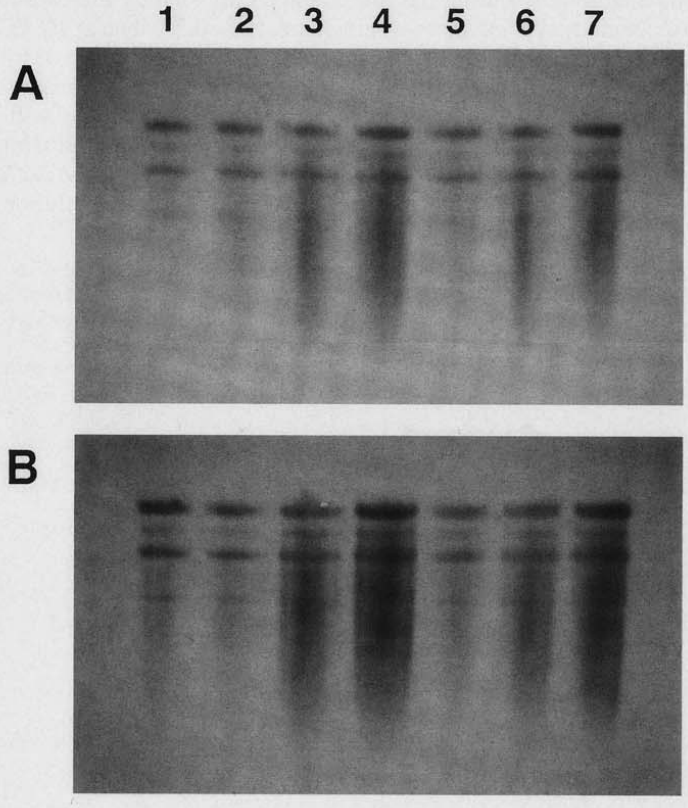

Fig. 5. Changes in ADH isozymes in carrot shreds held in 0.5% or 2.1% O_2 atmospheres and air at 5 (A) and 15 °C (B). Day 0 (lane 1); day 1, air (lane 2), 2% O_2 (lane 3), and 0.5% O_2 (lane 4); and day 3, air (lane 5), 2% O_2 (lane 6), and 0.5% O_2 (lane 7). Top is the cathode and bottom is the anode in each section.

The concentration of acetaldehyde was much lower than the corresponding concentration of ethanol but followed a similar pattern (Fig. 2). At day 3, the concentration of acetaldehyde in the carrots was 1.2%, 1.3%, 1.0%, and 1.1% of the corresponding ethanol concentration for 0.5% and 2% O_2 at 15 and 5 °C, respectively.

Endogenous production of acetaldehyde and ethanol is due to the role of PDC, which catalyzes the decarboxylation of pyruvate to acetaldehyde and is at a branch point between the Krebs cycle and ethanolic fermentation. The terminal enzyme in the ethanolic fermentation pathway is ADH that converts acetaldehyde to ethanol (Kennedy et al., 1992). The ADH and PDC activities in carrot shreds increased sharply under low O_2 and reached plateaus after 3 d, whereas they remained unchanged in air (Figs. 3 and 4). The ADH in shreds held in 0.5% and 2% O_2 at 15 and 5 °C increased to levels that were 7.6-, 3.8-, 6.3-, and 2.7-times greater, respectively, than those held in air. The PDC levels were 4.2-, 2.3-, 3.9-, and 2.2-times greater, respectively. The increase in ADH and PDC activities during the first 3 d were similar to the increase in ethanol and acetaldehyde increases (Figs. 1–4). A detailed time study with beet roots showed that the increase in ADH and PDC activities occurred within 3 h after transfer from air to anoxia (Zhang and Greenway, 1994).

Most diploid plants have multiple ADH genes (Gottlieb, 1982; Sachs and Ho, 1986), many having a two-gene, three-isozyme system (Tanksley, 1983; Xie and Wu, 1989) and some plants have more than two ADH genes [e.g., barley (Hanson et al., 1984) and pear (Nanos et al., 1992)]. However, all isozymes could not be found in each plant tissue due to the limited expression of the specific ADH isozymes (Ke et al., 1994; Xie and Wu, 1989). Extracts of carrot cells in suspension culture exhibited five bands of ADH activity on zymograms of starch gel electrophoresis; however, extracts of root tissues of mature carrot showed only three bands of ADH activity (Chourey and Widholm, 1980). Using polyacrylamide gel electrophoresis, four ADH isozemes were identified in extracts of carrot shreds (Fig. 5). One or two bands stained slightly more intensely in day-1 and -3 samples stored under low-O_2 atmospheres than samples stored in air. These results indicate that the increase in ADH activity may be due to increased synthesis of the enzyme. In maize, mRNA for ADH began to increase after 1.5 h of anaerobic treatment and continued to increase for 5 h when the level plateaued at a 50-fold above that of the aerobic level (Dennis et al., 1985).

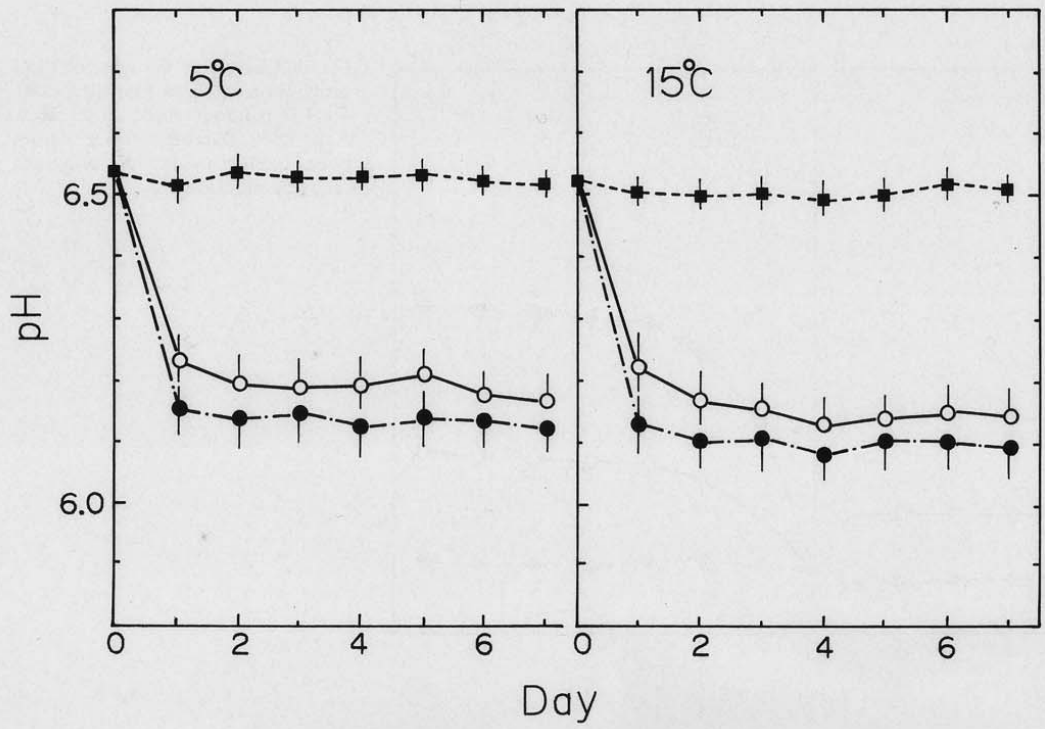

Fig. 6. Changes in pH in carrot shreds held in 0.5% (●) or 2% O_2 (○) atmospheres and air (■) at 5 and 15 °C. Data are means ± SE from three experiments with three assays for each determination.

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The pH of the carrot shreds kept in air remained constant at ≈6.5 (Fig. 6). Under low-O_2 atmospheres the pH dropped 0.3 to 0.4 units and remained at this level during the experiment. These pH values are similar to those reported in carrot disks (Leshuk and Saltveit, 1991). Roberts et al. (1984) reported that ADH played an important role for tight cytoplasmic pH regulation under hypoxia. This was shown with maize ADH null mutants that were unable to synthesize ethanol during anaerobic stress and also were unable to regulate cytoplasmic pH due to competing lactic acid fermentation, which led to cytoplasmic acidosis and premature death. The optimum pH for PDC and ADH present in crude extracts of carrots was ≈6 and 7, respectively, under the standard assay condition. A decrease in pH would activate PDC and slightly inhibit ADH activity; however, ADH activity was ≈10 times higher than PDC activity (Figs. 3 and 4). Therefore, a possible inhibition of ADH activity by a decrease in pH would not limit the conversion of acetaldehyde to ethanol, and the reduced pH may increase somewhat the ethanolic fermentation.

Low-O_2 atmospheres increased ethanol and acetaldehyde concentrations and the activities of ADH and PDC. All the increases were greater at 0.5% than 2% O_2 and at 15 than 5 °C. These results suggest that ethanolic fermentation was accelerated more in 0.5% than 2% O_2 and more accelerated at the higher storage temperature than the lower one. This acceleration may allow glycolysis to continue due to consumption of pyruvate and regeneration of NAD^+ (Davies, 1980; Kennedy et al., 1992). In addition, ADH was necessary for removal of acetaldehyde because of its phytotoxic effect (Perata and Alpi, 1991) and for tight cytoplasmic pH regulation (Roberts et al., 1984). When O_2 becomes limiting, glycolysis has accelerated in many plants, and the glycolytic pathway was considered to replace the Krebs cycle as the main source of energy (Kennedy et al., 1992). Thus, ethanolic fermentation may be essential for the plant's survival during O_2-limiting conditions. Carrot tissues showed an increase in glycolysis (Kato-Nogochi and Watada, 1996a, 1996b) and ethanolic fermentation under low-O_2 atmospheres and could produce some ATP through substrate phosphorylation, which may permit carrot tissues to survive temporarily without serious injury.

Literature Cited

Abdel-Rahman, M. and F.M.R. Isenberg. 1974. Effects of growth regulators and controlled atmosphere on stored carrots. J. Agr. Sci. 82:245–249.

Arulsekar, S. and D.E. Parfitt. 1986. Isozyme analysis procedures for stone fruits, almond, grape, walnut, pistachio, and fig. HortScience 21:928–933.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Carlin, F., C. Nguyen-The, G. Hilbert, and Y. Chambroy. 1990. Modified atmosphere packaging of fresh, "ready-to-use" grated carrots in polymeric films. J. Food Sci. 55:1033–1038.

Chourey, P. and J. Widholm. 1980. Tissue specific alcohol dehydrogenase isozyme variation in carrot: whole plant versus in vitro cultured cells. In Vitro 16:571–574.

Davies, D.D. 1980. Anaerobic metabolism and the production of organic acids, p. 581–611. In: D.D. Davies (ed.). The biochemistry of plants. vol. 2. Academic, New York.

Dennis, E.S., M.M. Sachs, W.L. Gerlach, E.J. Finnegan, and W.J. Peacock. 1985. Molecular analysis of the alcohol dehydrogenase 2 (*Adh2*) gene of maize. Nucleic Acids Res. 13:727–743.

Gottlieb, L.P. 1982. Conservation and duplication of isozymes in plants. Science 216:373–380.

Hansen, H. and G. Rumpf. 1974. Storage of carrots (variety Nantaise); the influence of the storage atmosphere on flavor, decay and content of sucrose, glucose and fructose. Acta Hort. 38:321–326.

Hanson, A.D., J.V. Jacobsen, and J.A. Zwar. 1984. Regulated expression of three alcohol dehydrogenase genes in barley aleurone layers. Plant Physiol. 75:573–581.

Kato-Noguchi, H. and A.E. Watada. 1996a. Regulation of glycolytic metabolism in fresh-cut carrots under low oxygen atmosphere. J. Amer. Soc. Hort. Sci. 121:123–126.

Kato-Noguchi, H. and A.E. Watada. 1996b. Low-oxygen atmosphere increases fructose 2,6-bisphosphate in fresh-cut carrots. J. Amer. Soc. Hort. Sci. 121:307–309.

Ke, D., E. Yahia, B. Hess, L. Zhou, and A.A. Kader. 1995. Regulation of fermentative metabolism in avocado fruit under oxygen and carbon dioxide stresses. J. Amer. Soc. Hort. Sci. 120:481–490.

Ke, D., E. Yahia, M. Mateos, and A.A. Kader. 1994. Ethanolic fermentation of 'Bartlett' pears as influenced by ripening stage and atmospheric composition. J. Amer. Soc. Hort. Sci. 119:976–982.

Kennedy, R.A., M.E. Rumpho, and T.C. Fox. 1992. Anaerobic metabolism in plants. Plant Physiol. 100:1–6.

Leshuk J.A. and M.E. Saltveit, Jr. 1990. Controlled atmosphere storage requirements and recommendations for vegetables, p. 315–352. In: M. Calderon and R. Barkai-Golan (eds.). Food preservation by modified atmospheres. CRC Press, Boca Raton, Fla.

Leshuk J.A. and M.E. Saltveit, Jr. 1991. Effects of rapid changes in oxygen concentration on the respiration of carrot roots. Physiol. Plant. 82:559–568.

Liew, C.L. and R.K. Prange. 1994. Effect of ozone and storage temperature on postharvest diseases and physiology of carrots (*Daucus carota* L.). J. Amer. Soc. Hort. Sci. 119:563–567.

Nanos, G.D., R.J. Romani, and A.A. Kader. 1992. Metabolic and other responses of 'Bartlett' pear fruit and suspension cultured 'Passe crassane' pear fruit cells held in 0.25% O_2. J. Amer. Soc. Hort. Sci. 117:934–940.

Perata, P. and A. Alpi. 1991. Ethanol-induced injuries to carrot cells: The role of acetaldehyde. Plant Physiol. 95:748–752.

Phan, C.T., H. Hsu, and S.K. Sarkar. 1973. Physical and chemical changes occurring in the carrot root during storage. Can. J. Plant Sci. 53:635–641.

Roberts, J.K.M., J. Callis, O. Jardetzky, V. Walbot, and M. Freeling. 1984. Cytoplasmic acidosis as a determinant of flooding intolerance in plants. Proc. Natl. Acad. Sci. USA. 81:6029–6033.

Sachs M.M. and T.-H.D. Ho. 1986. Alteration of gene expression during environmental stress in plants. Annu. Rev. Plant Physiol. 37:363–376.

Tanksley, S.D. 1983. Gene mapping, p. 109–138. In: S.D. Tanksley and T.J. Orton (eds.). Isozymes in plant genetics and breeding, part A. Elsevier Science Publishers, Amsterdam, The Netherlands.

Van den Berg, L. and C.P. Lentz. 1966. Effect of temperature, relative humidity, and atmospheric composition on changes in quality of carrots during storage. Food Technol. 20:954–957.

Wills, R.B.H., P. Wimalasiri, and K.J. Scott. 1979. Short term exposures to high carbon dioxide or low oxygen atmospheres for the storage of some vegetables. HortScience 14:528–530.

Xie, Y. and R. Wu. 1989. Rice alcohol dehydrogenase genes: Anaerobic induction, organ specific expression and characterization of cDNA clones. Plant Mol. Biol. 13:53–68.

Zhang Q. and H. Greenway. 1994. Anoxia tolerance and anaerobic catabolism of aged beet root storage tissues. J. Expt. Bot. 45:567–575.

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