Effects of Low Temperature on Ethylene Formation, Membrane Permeability and Fatty Acid Composition in Callus Derived from Apple (Malus pumila Mill. cv. Sensyu) Fruit

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Summary

The influence of low temperature on ethylene formation, membrane permeability, and free fatty acid composition were investigated in callus derived from 'Sensyu' apple (Malus pumila Mill.) fruits harvested at 71 days after full bloom (DAFB). 1-Aminocyclopropane-1-carboxylic acid (ACC) level, ethylene-forming enzyme (EFE) activity, and the rates of ethylene and carbon dioxide production in callus were stimulated by exposure to the low temperatures (−1°C, 0°C or 5°C). Electrolyte leakage and levels of free fatty acid in callus at chilling temperatures (−1°C, 0°C) for 2 days were higher than those of callus stored at non-chilling temperatures (5°C, 25°C). Thus, there seems to be a relationship between the increases in membrane permeability of callus chilled at 0°C or −1°C and the release of fatty acids from intact membrane lipids.

Introduction

It is known that low temperature disorders occur in apple fruit after storage for several months at 0°C ~ 4°C, although these disorders are cultivar-specific. For example, 'McIntosh' develops "corky" flesh browning (7), 'Starking Delicious' develops "internal breakdown" (6), and Australian 'Jonathan' develops both "soft scald" and "breakdown" (16).

According to our recent study, 'Sensyu' apple fruits develop "pitting" and "cracking", especially around the stalk cavity; "breakdown" secondarily occurs beneath the skin and proceeds towards the core after storage for 2 months at 0°C or −1°C (22).

The use of an in vitro model system supplies many advantages over intact tissue as an experimental tool to examine the mechanism of physiological injury in plants (15). Tissue culture can provide a homogenous system for metabolic studies and can be harvested throughout the year. Forney and Peterson (4) used callus derived from grapefruit albedo tissue to investigate the effect of preconditioning temperature on the rate of chilling-stimulated K⁺ leakage; Breidenbach and Waring (1) observed that tomato suspension cells and seedlings responded comparably when subjected to chilling stress. Lieberman et al. (8) reported ethylene production in apple fruit callus and suspension cells, but they did not study the influence of chilling temperatures.

The present study was undertaken to examine the response of callus derived from apple fruits to chilling stress, especially the effect of low temperatures on ethylene formation and membrane permeability, in order to ascertain if apple fruit callus would provide a suitable system for investigating the mechanism of chilling injury in intact apple fruits.

Materials and Methods

'Sensyu' apple fruits (Malus pumila Mill.) were picked on 9 July 1988 (71 DAFB) in the orchard of the University of Tsukuba. Fruits were momentarily washed with 70% ethanol, immersed in a 1% sodium hypochlorite solution for 10 min, and rinsed 3 times with sterile water. The cortical tissue was aseptically excises from the fruit, and 100 mg cubes were cultured individually in 100 ml Erlenmeyer flasks containing 25 ml of liquid culture.
medium that included Murashige and Skoog (11) mineral salts, 0.2 mg/liter, 2,4-dichlorophenoxyacetic acid (2,4-D); 0.03 mg/liter, benzyladenine (BA); 1 mg/liter, thiamin HCl; 0.5 mg/liter, nicotinic acid; 0.5 mg/liter, pyridoxin; 100 mg/liter, myoinositol; 2 mg/liter, glycine, and 30 g/liter sucrose. Media pH was adjusted to 5.7 using NaOH prior to autoclaving 15 min at 127°C. Cultures were maintained on a reciprocating shaker operated at 52 passes per min at 25°C under continuous fluorescent light (30 ᵉ⁴ Em⁻² sec⁻¹); calli were subcultured every month.

Six months after culture, callus was subjected to chilling (−1°C, 0°C) and non-chilling temperatures (5°C, 25°C) in the dark for 2 days. It was then removed from culture, weighed and used for determination of the rates of carbon dioxide and ethylene production, ethylene-forming enzyme (EFE) activity, 1-aminocyclopropane-1-carboxylic acid (ACC) content, electrolyte leakage, and free fatty acid content and composition.

Calli (0.7 g) were sealed in 50 ml Erlenmeyer flask containing 5 ml 10 mM MES buffer (pH 6.0) with or without ACC (0.2 mM) at 25°C for 3 hr on a reciprocatory shaker. The conversion of applied ACC to ethylene was used as a measure of EFE activity. One ml of head space gas in the flask was withdrawn by syringe for carbon dioxide and ethylene analysis with a Hitachi 163 gas chromatograph under the following conditions: column: Porapack Q 80-100 mesh, 3 mm × 2 m glass column; column temperature: 75°C; carrier gas: He 25 ml/min; detector: thermal conductivity detector for carbon dioxide, flame ionization detector for ethylene.

Calli were homogenized with 80% ethanol (35 ml/g tissue) for extraction of ACC in a mortar and pestle at 5°C for 12 hr. After centrifugation at 8000 × g for 10 min, the supernatant was concentrated to dryness in vacuo at 45°C in a rotary evaporator. The residue was dissolved in 3 ml of water, and an aliquot of 0.5 ml was assayed for ACC by the method of Lizada and Yang (9).

Electrolyte leakage was used as measure of chilling injury. Calli (0.7 g) were put into a 50 ml Erlenmeyer flask containing 25 ml 10 mM MES buffer (pH 6.0) on a reciprocating shaker at 25°C. After 3 hr, the tissue was removed from the MES buffer and the conductivity of the leachates was determined with a conductometer (CM-30ET, TOA Electronics Ltd.)

Calli (5 g) were homogenized with ten volumes of chloroform : methanol (2 : 1, v/v) in a mortar and pestle. 0.2 volume of water was mixed with the extracts, and the lower layer was collected and dried by nitrogen gas. The residue was dissolved in 1 ml of phenyltrimethyl ammonium hydroxide (PTAH, an on-column methylation reagent). An aliquot of 1 μl was analyzed for fatty acid by a gas chromatography / mass spectrometer (Shimadzu, GCMS-QP1000) under the following conditions: column packing, OV-17 80-100 mesh in a 2.6 mm × 1.1 m glass column; column temperature, 150°C to 210°C (2°C/min); carrier gas, He 30 ml/min; injection temperature, 265°C; ion source temperature, 250°C; ionizing voltage, 70 eV; molecular separator temperature, 270°C; and ionizing mode, EI.

**Results and Discussion**

The apple fruit callus chilled at 0°C and −1°C for 2 days became yellow, as compared to callus stored at 5°C and 25°C which remained green (Data not shown).

Changes in respiration rate are often used as an indicator of stress. In this study, the rate of carbon dioxide production in callus was higher at exposures to 5°C, 0°C and −1°C, as compared to 25°C (Fig. 1), suggesting that a relation exists between respiration and chilling sensitivity. We found a similar relationship in kiwifruit during the chill-storage, in that the respiration rate tended to increase before the occurrence of chilling injury symptoms (21). Yamawaki et al. (23) reported a functional abnormality of cell mitochondria and a

![Fig. 1. Carbon dioxide production in apple fruit callus stored at 25°C, 5°C, 0°C or −1°C for 2 days. Means of three samples ± SE.](image-url)
degeneration of cell membrane permeability occurred before symptoms of chilling injury became visible.

Chilling-induced ethylene production has been described for other plant tissues (2, 3, 20). In this investigation, ethylene production was induced by exposure to the low temperatures (−1°C, 0°C or 5°C) but ACC level was only slightly higher in chilled callus, as compared to callus held at 25°C. EFE activity, however, was higher at the lower temperatures (Fig. 2), which suggests that the increase in ethylene evolution is due to the enhanced EFE activity in chilled callus. Wang and Adams (20) reported that ethylene production in cucumbers increased during the first 4 days of chilling, but the rate declined to the lowest level after 7 days of exposure. Damage to the EFE system in cucumbers was cited as the cause for the decline in the rate of ethylene production upon prolonged chilling.

Electrolyte leakage is a parameter that has often been used to indicate physical damage to the plasma membrane as a result of low temperature stress (19). The callus chilled at 0°C and −1°C in this study showed a marked increase in electrolyte leakage, as compared to 25°C and 5°C (Fig. 3). Paull (14) found an increase in leakage rate within 1 hr of exposing chilling-sensitive tissue in chilling temperatures. He considered cell leakage as an early symptoms of changes in the membrane of chilling-sensitive plants.

Fig. 2. ACC content, EFE activity and ethylene production in apple fruit callus stored at 25°C, 5°C, 0°C or −1°C for 2 days. Means of three samples ± SE.

Fig. 3. Electrolyte leakage, ratio of unsaturated/saturated fatty acids (ratio of UFA/SFA) and levels of free fatty acid (FFA content) in apple fruit callus stored at 25°C, 5°C, 0°C or −1°C for 2 days. Means of three samples ± SE.
Many of the theories on chilling injury are in one way or another centered on membranes, primarily the plasma membrane. An enrichment in membrane phospholipid content with increasing cold hardiness has been demonstrated in a wide range of plants (24). Lyons (10) reported a correlation between the contents of unsaturated fatty acid and the chilling injury tolerance of plants and fruits. In our study, the abundant fatty acids in apple fruit callus were palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). The linoleic and linolenic acids decreased in callus stored at 5°C, as compared to 25°C. The decrease in linoleic and linolenic acids were inverse to the increase in oleic acid and stearic acid (Table 1). The ratio of unsaturated vs. saturated fatty acids decreased in callus held at 5°C for 2 days. However, when the temperature was lower than 5°C, this ratio showed an increase (Fig. 3). The levels of free fatty acid showed little difference in callus exposed at 5°C and 25°C. Whereas, there was an increase of free fatty acid levels in callus chilled at 0°C and −1°C, as compared to 25°C (Fig. 3). Gemel and Kaniuga (5) reported an increase in free fatty acid levels in chloroplasts isolated from cucumber leaves chilled for 3 to 4 days in the dark. Free fatty acids also accumulate in the microsomal fraction of leaves of tomato plants chilled in the presence of light (17). It is unlikely that this effect is induced primarily by illumination. In cucumber fruit, 3- to 7-day exposure at 4°C resulted in an increase in lipid peroxidation which took place before irreversible injury occurred; phospholipase D activity appeared to be potentiated when fruits were rewarmed after 7 days of chilling (13). In addition, membrane lipid peroxidation also causes phospholipase A activity (18) and membrane fatty acid deesterification (12). Although we did not assay for the enzyme, our results indicated that some lipolytic activities are potentiated in callus chilled at 0°C and −1°C. Therefore, we presume that a relationship exists between the increase in the level of free fatty acids and the release of fatty acids from intact membrane lipids in apple fruit callus chilled at 0°C or −1°C for 2 days.

Apple fruit callus tissue provides a convenient experimental system for studying the physiological and biochemical responses of apple fruit to chilling stress throughout the year. The advantages of this system should assist research efforts in investigating the mechanism of chilling injury in intact apple fruits.

Acknowledgments

We wish to thank Miss Madoka Kurokawa for excellent technical assistance.

Literature Cited

リンゴ‘千秋’果実から誘導されたカルスにおけるエチレン生成、膜透過性ならびに脂肪酸組成に及ぼす低温の影響

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摘要

摘開71日後のリンゴ（Malus pumila Mill．‘千秋’）果実から誘導されたカルスにおけるエチレン生成、膜透過性ならびに脂肪酸組成に及ぼす低温の影響を調査した。ACC含量、EFE活性、エチレン生成量ならびに呼吸活性が低温処理（－1℃～5℃）によって著しく増加する傾向が認められた。5℃あるいは25℃に比べて、障害発生温度域である0℃または－1℃に2日間おいたカルスは、電解質浸出度および遊離脂肪酸の含量が高かった。すなわち、0℃または－1℃においたカルスにおける膜透過性の増加と遊離脂肪酸の含量の増加との間に密接な関係があると思われた。