Volatile Emissions and Chlorophyll Fluorescence as Indicators of Freezing Injury in Apple Fruit

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Abstract. Use of volatile emissions and chlorophyll fluorescence as indicators of freezing injury were investigated for apple fruit (Malus ×domestica Borkh.). ‘Northern Spy’ and ‘Delicious’ apples were kept at –8.5°C for 0, 6, or 24 h, and then at 20°C. After 1, 2, 5, and 7 d at 20°C, fruit were analyzed for firmness, skin and flesh browning, soluble solid content, titratable acidity, ethanol, ethyl acetate, ethylene, respiration rate, and chlorophyll fluorescence. Freezing caused skin and flesh browning and a loss of fruit firmness, which was greater in ‘Northern Spy’ than in ‘Delicious’. In ‘Northern Spy’ fruit subjected to the freezing treatments, ethanol and ethyl acetate concentrations were as much as 37- and 300-fold greater, respectively, than in control fruit. ‘Delicious’ fruit showed similar patterns of ethanol and ethyl acetate increase, but of lower magnitude, as a result of freezing. Higher fruit respiratory quotients were associated with increased ethanol and ethyl acetate concentrations. Ethylene production and chlorophyll fluorescence of fruit were reduced by freezing.

Freezing injury of apple fruit can occur on the tree because of extreme weather, in storage because of improper conditions, or in transit as a result of improper handling. The influence of freezing on fruit quality is variable and dependent on the degree and duration of freezing stress. Symptoms of severe injury, following inter- and intra-cellular freezing, include firmness loss, skin and flesh browning, water-soaked flesh, shriveling, and development of off-flavors (Pierson et al., 1971). In mild-to-moderate freezing injury, visual symptoms are often not apparent; however, increased softening and decay may reduce storage life (Bir and Bramlage, 1973; Carrick, 1924; Smock, 1970). Fruit that have been frozen must be identified to avoid storing them.

Volatile compounds, such as ethanol, acetaldehyde, and ethylene, have been reported to be indicators of plant injury from freezing, heat stress, water stress, and SO2 fumigation (Forney and Jordan, 1998; Kimmerer and Kozlowski, 1982; Templeton and Colombo, 1995). Indications of physiological damage include elevated concentrations of ethanol in heat-treated fresh broccoli (Brassica oleracea L. Italica group) (Forney and Jordan, 1998) and loss of viability in stressed tree seedlings (Templeton and Colombo, 1995). Volatile emissions from apple fruit have been related to low oxygen stress (Mattheis et al., 1991a) and damage from high concentrations of carbon dioxide (Volz et al., 1998), but not to freezing damage.

In apple fruit, chlorophyll fluorescence is a useful indicator of several postharvest stresses, including exposure to low O2 or high CO2 concentrations (DeEll et al., 1995; 1998). Chlorophyll fluorescence has also been used as an indicator of chilling stress in green peppers (Capsicum annuum L.) (Lurie et al., 1994), cucumbers (Cucumis sativus L.) (van Kooten et al., 1992), banana (Musa acuminata Colla), and mango (Mangifera indica L.) (Smillie et al., 1987).

Indicators of freezing stress could be useful for identifying freeze-damaged apple fruit that may have a shortened storage life. Changes in volatile emissions and/or chlorophyll fluorescence of fruit could serve as stress indicators. Therefore, the objectives of this study were to: 1) determine the effects of freezing stress on production of stress-induced volatiles, including ethanol, ethyl acetate, and ethylene; 2) characterize freezing-induced changes in chlorophyll fluorescence; and 3) relate these stress indicators to changes in fruit respiration and quality.

Material and Methods

Freezing treatment. Apple fruit, cvs. Northern Spy and Delicious, were obtained from commercial orchards, graded, and stored at 0°C in air for ≥1 month prior to treatment. Three 46-L plastic containers for each cultivar were each filled with ≥100 apples, placed in a –8.5 ± 0.5°C room with one container of each cultivar being removed after 0, 6, or 24 h. The 0 h fruit served as the control, having never been exposed to freezing temperature. Following the freezing treatment, containers of fruit were kept at 20°C until analyzed. Temperature was monitored in three fruit of each cultivar during the 24-h freezing period by placing thermocouples in the cortex.

Fruit quality. For evaluation of fruit quality, samples of 10 apples were taken for each cultivar and freezing treatment 1, 2, 5, and 7 d after freezing. Firmness was determined on opposite sides of each fruit after peel removal using a mounted Ballauf penetrometer (Ballauf Manufacturing Co., Laurel, Md.) with an 11.1-mm-diameter tip. Juice expressed during firmness testing was analyzed for percentage of soluble solids content with a hand-held temperature-compensated refractometer (Atago Co., Tokyo), and for titratable acidity by titrating a 2-mL juice sample with 0.1 mol·L–1 NaOH using a semi-automatic titrator (Multi-Dosimat E-415 titrator; Metrohm AG, Herisau, Switzerland) to a phenolphthalein endpoint of pH 8.1. After cutting in half equatorially, each of the 10 apples was rated for peel and cortex browning using a scale of 0–3, where 0 = none, 1 = slight, 2 = moderate, and 3 = severe browning.

Volatile analysis. Two subsamples of six apples each were sampled for volatile emissions by sealing the whole fruit in 4-L glass jars with Teflon® lids 1, 2, 5, and 7 d after transfer to 20°C. The headspace over the apples was allowed to equilibrate for 1 h under a 100 mL·min–1 flow of purified air, following which a 100 mL sample of the jar headspace was trapped onto 120 mg of Tenax GR 20/35 (Alltech Associates, Deerfield, Ill.) in a 100 mm × 6.4 mm (o.d.) glass tube. Samples were analyzed on a Magnum gas chromatograph-mass spectrometer (GC-MS) system (Finnigan MAT, San Jose, Calif.) equipped with an LSC 2000 purge and trap concentrator (Tekmar, Cincinnati) with the valve and transfer line being held at 170°C. Traps were placed in the concentrator and desorbed at 250°C for 4 min directly onto a Supelcowax 10 (Supelco, Bellefonte, Pa.) column (60 m × 0.53 mm) with a film thickness of 1 μm. Column flow rate was 10 mL·min–1 of helium while temperatures of the transfer line from the GC to the MS and the MS were 180 and 220°C, respectively. Column temperature was held at 40°C for 5 min, increased to 50°C at a rate of 2°C·min–1, increased to 200°C at a rate of 40°C·min–1.
of 5 °C·min⁻¹, and held at 200 °C for 5 min. Quantitation of ethanol and ethyl acetate was performed using single ions of external standards. All peak areas were normalized using the peak area of a 4 ng dodecanec standard that was run on the day of the analysis.

Respiration and ethylene production. Two 10-apple samples for each cultivar and freezing treatment were sealed in 4-L plastic jars and held at 20 °C. Air flow (20 mL·min⁻¹) was maintained through each jar. One mL samples of outflow air were taken 1, 2, 5, and 7 d after freezing treatment and analyzed for O₂, CO₂, and ethylene. Oxygen and CO₂ were measured using a Varian 3400 gas chromatograph with a thermal conductivity detector (Varian Associates, Walnut Creek, Calif.) and a 1.9 m × 6.4 mm (o.d.) CTR I column (Alltech Associates). Respiratory quotient (RQ) was calculated as CO₂ production rate divided by the O₂ consumption rate. Ethylene was analyzed using a gas chromatograph with a flame ionization detector (Carle Instruments, Anaheim, Calif.). Samples were injected into a 1.9 m × 3.2 mm (o.d.) activated alumina column with a helium carrier flow of 50 mL·min⁻¹, and ethylene was quantified by comparison with certified standards.

Chlorophyll fluorescence. Chlorophyll fluorescence was determined using a plant productivity fluorometer (model SF-20; Richard Brancen Research, Ottawa, Ont., Canada). Twenty apples of each cultivar were sampled 1, 2, 5, and 7 d after removal from the freezing treatment. Apples were dark-adapted by placing them in the dark at room temperature for 4 h before fluorescence measurements were taken. With a light intensity of 20 mmol·m⁻²·s⁻¹, the fluorometer probe was placed firmly on one side of the fruit. The origin (Fo), peak (Fp) and terminal (Ft) values were recorded from the fluorometer digital display; the Ft value was obtained after 50 s. Variable fluorescence (Fv) was calculated as Fv = Fp – Ft. The Fo obtained from this type of fluorometer is not very accurate, and thus the more common calculation (Fv = Fp – Fo) could not be used.

Statistical analysis. The experimental design was a completely randomized 2 × 3 × 4 factorial design, with two cultivars, three freezing durations, and four evaluation times. For each combination, two 6- or 10-fruit samples were used for analysis of volatiles and respiration, respectively, while 20 or 10 fruit were used for fluorescence and quality measurements, respectively. The two cultivars were analyzed separately for volatiles, respiration, and fluorescence. Data were analyzed using the analysis of variance (ANOVA) and LSD options of Genstat 5 (Genstat 5 Committee, 1993).

Results

Freezing temperatures. Apples of both cultivars cooled rapidly in the –8.5 °C room. ‘Northern Spy’ and the ‘Delicious’ apples super-cooled to –3.3 and –2.5 °C, respectively. The first exotherm, indicating intracellular ice formation, was observed after 2–5.5 h at –8.5 °C. Following the exotherm, fruit temperature held constant at –1.7 °C in both cultivars for ≈10 h and then decreased slowly. After 24 h, fruit temperature of ‘Northern Spy’ and ‘Delicious’ apples averaged –4.2 and –2.3 °C, respectively.

Fruit quality. Firmness of apples treated for 24 h was 30% and 10% less than that of controls for ‘Northern Spy’ and ‘Delicious’, respectively (Table 1). Firmness of fruit treated for 6 h was less influenced than that of those held for 24 h, but they were softer than controls. Some softening occurred during 7 d at 20 °C (data not shown); however, most of the freezing-induced firmness loss was apparent 1 d following treatment. Peel and cortex browning also increased with longer freezing times. Browning was greater in ‘Northern Spy’ than in ‘Delicious’ fruit. Both peel and cortex browning were apparent 1 d after treatment and did not change significantly during 7 d at 20 °C. Soluble solids were not influenced by freezing. The effect on titratable acidity (TA) varied with cultivar. Titratable acidity in ‘Northern Spy’ fruit from the 24 h treatment was 12% lower than that in control fruit, but was 15% higher than that of the controls in ‘Delicious’.

Table 1. Quality measurements of ‘Northern Spy’ and ‘Delicious’ apples after exposure to –8.5 °C for up to 24 h, followed by 1, 2, 5, and 7 d at 20 °C. Data are means for all times at 20 °C. F probabilities for the effects of cultivar (C), treatment time (T), C × T, and days at 20 °C were derived from the ANOVA.

| Cultivar          | Time at –8.5 °C (h) | Firmness (%) | Peel browning rating | Cortex browning rating | Soluble solids (%) | Titratable acidity (%) |
|-------------------|---------------------|--------------|----------------------|------------------------|-------------------|------------------------|
| Northern Spy      |                     |              |                      |                        |                   |                        |
| 0                 | 75.6                | 0.02         | 0.00                 | 13.8                   | 1.69              |
| 6                 | 68.5                | 0.18         | 0.52                 | 14.0                   | 1.71              |
| 24                | 52.1                | 1.10         | 1.40                 | 13.5                   | 1.48              |
| Delicious         |                     |              |                      |                        |                   |                        |
| 0                 | 63.6                | 0.00         | 0.00                 | 14.0                   | 0.80              |
| 6                 | 61.4                | 0.00         | 0.08                 | 14.3                   | 0.88              |
| 24                | 56.9                | 0.15         | 0.32                 | 14.4                   | 0.92              |

- 0 = none, 1 = slight, 2 = moderate, and 3 = severe.
- Percentage of malic acid.
- n = 4, df = 23.

Fig. 1. (A and B) Headspace ethanol and (C and D) ethyl acetate concentration above ‘Northern Spy’ and ‘Delicious’ apples held at –8.5 °C for 0, 6, or 24 h, followed by 7 d at 20 °C. Vertical bars represent LSD at P ≤ 0.05.
Volatile emissions. One day after fruit were removed from freezing conditions, ethanol concentration in the headspace was 37-fold greater for ‘Northern Spy’ apples that were held at –8.5 °C for 24 h than for the control fruit (Fig. 1A). Ethanol emission from frozen fruit increased during the first 5 d at 20 °C but then remained constant from day 5 to day 7, being 5- and 3-fold greater than from control fruit, respectively. Ethanol emission was 2- to 4-fold greater for ‘Northern Spy’ fruit held at –8.5 °C for 6 h than from control fruit for the first 2 d following freezing. However, after 5 d at 20 °C, no differences between the 6 h-treated fruit and the controls were evident. After 5 and 7 d at 20 °C, headspace ethanol concentration in the control fruit was 23- and 37-fold greater than at 1 d, respectively.

Ethanol production of ‘Delicious’ was less affected by freezing than was that of ‘Northern Spy’ (Fig. 1B). Overall, headspace ethanol concentrations were 5- to 8-fold greater for ‘Northern Spy’ fruit subjected to the 24 h freezing treatment than for similarly treated ‘Delicious’ fruit. One and 2 d after freezing, headspace ethanol concentrations for ‘Delicious’ apples held for 24 h at –8.5 °C were similar to the controls (Fig. 1B), but the concentrations were about 5- and 3-fold greater than for controls after 5 and 7 d at 20 °C, respectively. Concentrations in the control fruit and those held at –8.5 °C for 6 h did not change during the 7 d at 20 °C.

Gas chromatography of the control headspace indicated the production of ethyl acetate and ethylene, as well as changes in other volatile emissions. Ethyl acetate was produced in greater amounts in ‘Northern Spy’ fruit than in controls at day 1 following treatment, while 5 d after treatment the difference was more than 30-fold (Fig. 1D). No differences were observed between the 6 h treatment and the control. Emissions from fruit treated for 24 h were 3- to 11-fold greater in ‘Northern Spy’ than in ‘Delicious’.

Unlike production of ethanol and ethyl acetate, ethylene production was reduced by freezing treatments in both cultivars, with the greatest reduction (>85%) occurring 2 d after treatment in apples held at –8.5 °C for 24 h (Fig. 2A and B). Ethylene production by these fruit increased 7 d after treatment but still remained 40% to 60% less than for control fruit. Ethylene evolution from fruit treated for 6 h was 20% to 50% less for controls after 1 or 2 d at 20 °C. However, this difference was <12% after 5 or 7 d.

Respiration. The RQ of ‘Northern Spy’ fruit subjected to the 24 h freezing treatment was substantially greater than that of the controls, while RQ increased only slightly in ‘Delicious’ (Fig. 3). The RQ of both cultivars decreased during the week at 20 °C, approaching control values after 7 d. In both cultivars, the RQ of fruit treated for 6 h was greater than that of controls after 1 d, but little difference was evident after 2 d at 20 °C.

Chlorophyll fluorescence. The Fv of ‘Northern Spy’ apples was consistently higher than that of ‘Delicious’ (Fig. 4). ‘Northern Spy’ fruit kept at –8.5 °C for 24 h had a lower Fv during the subsequent 7 d at 20 °C than did control fruit or those kept at –8.5 °C for 6 h (Fig. 4A). The Fv remained relatively constant for ‘Northern Spy’ apples kept at –8.5 °C for 24 h, whereas the Fv of the control fruit and those kept at –8.5 °C for 6 h increased slightly between 1 and 2 d at 20 °C and then gradually decreased. The Fv of ‘Delicious’ apples was not affected by freezing, although fruit held at –8.5 °C for 24 h tended to have the lowest Fv after 2, 5, and 7 d at 20 °C (Fig. 4B).

Discussion

Physiological indicators of injury could be useful for identifying fruit damaged by freezing or other stresses. In response to freezing stress, changes were observed in emissions of ethanol, ethyl acetate, and ethylene, as well as in fruit respiration rates and chlorophyll fluorescence. These nondestructive measurements could be used to identify stressed fruit with reduced quality or storage life.

Volatile emissions. Freezing stress stimulated ethanol production in apple fruit, which was associated with loss of firmness and development of skin and flesh browning. As in other studies, ‘Northern Spy’ was more sensitive than was ‘Delicious’ to freezing stress (Smock, 1970). The high concentration of ethanol produced by apples held for 24 h at –8.5 °C made them easy to differentiate from controls. Fruit held for 6 h at –8.5 °C, which developed less severe injury symptoms than those held for 24 h, produced more ethanol for the first 2 d following treatment, but not after 5 d. ‘Delicious’ fruit, which are more resistant to freezing injury (Smock, 1972), produced much less ethanol than did ‘Northern Spy’ fruit. In fruit frozen for 24 h, which developed slight injury, no difference in ethanol evolution was observed in the first 2 d following freezing, but evolution was greater than in controls after 5 d. In addition to the apparent cultivar differences in freezing sensitivity, ‘Northern Spy’ fruit were 2 °C colder than ‘Delicious’ fruit after 24 h of exposure to –8.5 °C, which may have contributed to the difference in injury and volatile emission.

The use of ethanol as an indicator of freezing injury in apples could be compli-

![Fig. 2](image-url) Ethylene production rate of (A) ‘Northern Spy’ and (B) ‘Delicious’ apples held at –8.5 °C for 0, 6, or 24 h, followed by 7 d at 20 °C. Vertical bars represent LSD at P ≤ 0.05.
cated by variability in rates of ethanol production in nonfrozen fruit. Variation in ethanol production may occur during normal ripening or senescence (Mattheis et al., 1991b). In ‘Northern Spy’ apples, ethanol emission from control fruit increased during 7 d at 20 °C to levels similar to those of fruit held at –8.5 °C for 24 h, then 1 d at 20 °C. However, ethanol emissions from control ‘Delicious’ fruit did not change with time. Therefore, to identify and interpret stress-induced increases in ethanol production, a better understanding is needed of ethanol production in nonstressed fruit during storage and ripening.

Ethanol is a fermentation product that occurs in plant tissues when O2 is limiting or cannot be utilized for oxidative phosphorylation. Freezing of plant tissues is a complex process causing many physical and chemical changes in the plant cell (Mazur, 1969), and can result in disruption of normal aerobic respiration. During freezing of apple, ice crystals form in intercellular spaces and water is drawn from the cells as the ice crystals increase in size. Cellular injury results from physical damage caused by the ice crystals as well as from dehydration of the cells, which can cause altered metabolic activity. Events such as cell shrinkage, reduction in spatial separation of macromolecules, and reduction in cell water content may inhibit utilization of O2, resulting in fermentation. The increase in the RQ associated with stress-induced ethanol production is also an indicator that fermentation is occurring in the fruit. During fermentation, CO2 is evolved without consumption of O2. Carrick (1928) reported that CO2 evolution was stimulated for several days following thawing of frozen ‘Winesap’ apples.

Ethyl acetate production was induced in a manner similar to that of ethanol except that differences between control and stressed apples were greater. Ethanol and acetyl CoA are substrates for ethyl acetate biosynthesis (Ke et al., 1994). The dramatic increase in production of ethyl acetate from ‘Northern Spy’ fruit may be attributed to the high levels of ethanol. Larsen and Watkins (1995) reported that ethanol and ethyl acetate were the major volatiles produced during fermentation of strawberry (Fragaria ×ananassa Duch.) fruit.

In this study, ethylene production was reduced as freezing injury increased. However, since rates of ethylene production by mature apples are highly variable depending on stage of ripeness (Dilley, 1969), ethylene may not be a good indicator of freezing stress. Determining whether ethylene production of apple fruit is reduced by freezing would be difficult without a nonfrozen control. Reduction in ethylene production caused by freezing may be the result of cellular damage. Bruising of ‘Golden Delicious’ apples reduced ethylene production (Robitaille and Janick, 1973). While freezing does not appear to induce ethylene production in apple, the response of other plant species to freezing is variable. Kimmerer and Kozlowski (1982) demonstrated that ethylene production decreased in Cucurbita maxima Duchesne and Brassica oleracea L., and increased in Quercus macrocarpa Michx. and Pinus halepensis Mill. seedlings following freezing of 30% of their leaf area. However, a poor correlation between stress and ethylene production was found when stress resulted in death of cells. In addition to the volatiles measured in this study, other stress-induced volatiles, such as acetaldehyde, propanol, hexanol, and the hexenols could also serve as stress indicators as reported in other plants (Forney and Jordan, 1998; Kimmerer and Kozlowski, 1982).

Chlorophyll fluorescence. The higher Fv in ‘Northern Spy’ apples was largely due to higher Fp values and hence greater differences in Fp-Ft, suggesting that ‘Northern Spy’ apples had more photosynthetic competence per unit of chlorophyll, and thus greater photosystem II activity (Smillie et al., 1987), than did ‘Delicious’ fruit. ‘Northern Spy’ apples held at –8.5 °C for 24 h had lower Fv during the subsequent 7 d at 20 °C than the control fruit or those held at –8.5 °C for 6 h (Fig. 4A). A similar trend was observed in ‘Delicious’ apples, although fluorescence was not affected by treatment (Fig. 4B). Similar reductions in chlorophyll fluorescence have been found in other plant tissues subjected to freezing stress (Strand and Öquist, 1988; Sundbom et al., 1982), and were attributed to reduced photochemical efficiency of photosystem II, resulting from an inhibition of electron flow from the primary electron acceptor of photosystem II (Qx) to the plastoquinone pool (Strand and Öquist, 1988).

Prange et al. (1997) found that Fv/Fm [Fv = Fm (max. fluorescence) – Fo (min. fluorescence)] decreased in apples as ethanol production rate increased, suggesting that ethanol accumulation in plant tissue reduced excitation energy transfer of photosystem II in the thylakoid membranes. Similar trends were observed in our experiment, as ‘Northern Spy’ apples held at –8.5 °C for 24 h had lower Fv (Fig. 4A) and produced more ethanol (Fig. 1A) during the subsequent 7 d at 20 °C, than did control fruit or those held at –8.5 °C for 6 h.

Conclusions

The increase in ethanol and ethyl acetate production, and the reductions in ethylene production and chlorophyll fluorescence, were associated with freezing damage in treated apples. Limited amounts of ethanol and ethyl acetate were evolved from nonstressed apples, but the amounts increased rapidly following a freezing stress, and could prove to be useful indicators of such stress.

Fig. 3. Respiratory quotient of (A) ‘Northern Spy’ and (B) ‘Delicious’ apples held at –8.5 °C for 0, 6, or 24 h. Following the freezing treatment, fruit were held at 20 °C under a 20 mL·min⁻¹ constant flow of humidified air. Vertical bars represent LSD at P ≤ 0.05.
The decrease in chlorophyll fluorescence was also closely related to stress-induced ethanol production and freezing injury. To determine the usefulness of stress-induced volatiles and fluorescence changes in identifying apples damaged by freezing or other stresses, additional research is needed to understand the effects of various pre- and postharvest conditions on these stress indicators.

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