Morphological and Physiological Changes during Maturation of New Mexican Type Peppers

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Abstract. New Mexican Chile peppers (Capsicum annuum L. ‘New Mexico 6-4’) were harvested at weekly intervals beginning 20 days after flowering (DAF), and were evaluated for ethylene (C2H4) production, respiration rates, chlorophyll content, degradative enzyme activity (cellulase, polygalacturonase, β-galactosidase), and fruit firmness. Morphological and physiological changes were most apparent in peppers harvested 54 to 69 DAF. β-galactosidase activity increased rapidly beginning 54 DAF and reached a peak by 89 DAF. Fruit firmness was highest (36 newtons) at 54 DAF and had decreased significantly by 69 DAF. Carbon dioxide production and chlorophyll content were highest in young pods harvested 20 DAF and decreased steadily thereafter. A climacteric increase of CO2 was absent. There were two peaks in C2H4 production: one associated with rapid fruit growth and the other with color change (61 to 69 DAF). Fruit harvested on the same day but at different developmental stages (green to red) were similar to those observed in fruit harvested over the season for the physiological characteristics tested. Separation of pepper fruit soluble proteins on SDS-PAGE demonstrated increased intensity in protein bands at 27, 35, and 40 kDa and decreased intensity of 51 kDa band as the fruit matured. Several biochemical processes appeared to be enhanced in Chile pepper fruit from 47 to 69 DAF.

Materials and Methods

Plant material. ‘New Mexico 6-4’ peppers were planted on 1 May 1991, in a field plot south of Las Cruces, N.M. The plots were cultivated and maintained according to local practices. Flowers were tagged at anthesis (24 July 1991), and pepper fruit were harvested weekly beginning 13 Aug. 1991. In addition, pepper fruit at developmental stages from immature green to red were harvested from plants on 11, 18, and 25 Sept., and physiological characteristics were measured.

Ethylene and CO2, C2H4, and CO2 were measured weekly. Four replications with three pods per replication were used for each measurement. Volume and weight were determined for each fruit. Volume was determined by submerging the fruit in water and measuring displacement. Fruit were placed in 2-liter, gas-tight jars with a rubber septum in the lid to allow gas sample withdrawal. Mean fruit weight varied from $\approx 20$ to $60$ g, depending on developmental stage (Fig. 1). Average time from harvest to sealing of the jars was $\approx 1$ h. Jars were kept at room temperature under fluorescent room lights.

Samples (1 ml) were withdrawn at hourly intervals for 8 h. Percentage of CO2 was determined using an infrared gas analyzer (Illinois Instruments, McHenry, Ill.) calibrated with a 1.0% CO2 standard.

Ethylene production was determined with a Hewlett Packard 5840A gas chromatograph equipped with an alumina column and flame ionization detector. Samples (1.0 ml) were collected 24 h after sealing the jars. A gas standard containing 10.5 nl C2H4/liter was used for calibration.

Firmness analysis. The force required to puncture the chile fruit was measured in three locations on the fruit: near the stem end, center, and tip of the pod. Firmness measurements for the three

Abbreviations: DAF, days after flowering; C2H4, ethylene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DNS, dinitrosalicyclic acid.

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locations were averaged to obtain a value for each fruit. Three fruit were tested from each of four replications. The firmness tests were performed with a Wagner (Wagner Instruments, Greenwich, Conn.) Force Dial model FDN 100 with a 6.5-mm probe, and the values recorded in newtons.

Chlorophyll assay. The chile fruit used for firmness measurements were also used to measure for chlorophyll. Six pairs of 8-mm disks were cut from adjacent sides of each chile to be similar in weight as possible. Disk pairs were cut from the top, middle, and bottom of each chile. One side (top, middle, and bottom) of the disks was used to determine milligrams of chlorophyll per gram fresh weight, and the other disks were used to determine milligrams of chlorophyll per gram dry weight. Chlorophyll content was determined with a spectrophotometer (Abeles et al., 1989).

Fruit color. Chile fruit at four stages of ripeness were harvested 18 Sept. 1991, and divided into five replications of four pods each. Maturity stages were characterized according to fruit color. Peppers in the green (G) ripeness group were fully developed pods, showing no orange or red pigmentation, and considered ready for green pepper harvest. Peppers developed to this stage by 30 to 40 days after flowering (DAF). The green/red (G/R) group was 10% to 20% orange or red, whereas the red/green (R/G) group was 50% to 60% orange or red. Color development began ≈ 47 to 54 DAF. The fourth group contained chile fruit that were completely red (R). In 1991, fruit were completely red by 68 DAF. Visual color was quantified using a Minolta CE-200 Chromameter (Minolta, Ramsey, N.J.) reading in the L,a,b mode. The L values express the lightness or darkness of colors, with lighter colors having higher L values. The a : b ratio represents the green to red (a) color range divided by the blue to yellow range (b). Samples with high a/b values are reddest. Color was measured in four locations to obtain an accurate representation. These measurements were repeated on chile fruit harvested 1 week later (25 Sept. 1991).

Enzyme extraction and gel electrophoresis. Pepper fruit were stored at -40°C until used for enzyme assays. Acetone powders were prepared from pepper fruit as described by Abeles and Biles (1990). In brief, 10 g of frozen pepper tissue were homogenized in ≈ 30 ml of aceton at room temperature. The homogenate was filtered through Whatman #1 filter paper on a Buchner funnel and washed with acetone at least three times. The powder was air dried at room temperature and stored at 20°C. Protein was extracted with distilled deionized water (24°C) for proteins used in gel electrophoresis or 0.05 M acetate buffer (pH 5) for enzyme assays. The buffer and acetone powder paste (10 buffer : 1 powder) was passed through miracloth (Chicopee Mills, N.Y.), and the extract was centrifuged for 5 min at 15,000× g. Extracts used for gel electrophoresis were filtered through a 0.22-µm filter after centrifugation. Proteins were concentrated through addition of 100 µl of 100% trichloroacetic acid (TCA) to 900 µl of extract. The precipitated proteins were washed once in TCA and twice in 100% acetone. Precipitated proteins with loading buffer were heated for 3 min at 100°C. Total protein (4 µg/lane) was separated on 4% stacking and 12% separation SDS-PAGE slab gels (0.75 mm thick) using the Laemmli (1970) buffer system, according to the instructions provided by BioRad (Richmond, Calif.). Gels were electrophoresed with 200 V (constant voltage) until the bromophenol tracking dye front moved off the gel and stained with colloidal coomassie brilliant blue G-250, according to Neuhoff et al. (1988). BioRad low molecular weight protein standards were used to estimate protein size. Protein (of pepper extracts) was measured with the BioRad protein dye method.

β-galactosidase activity was determined according to Gross et al. (1986). The substrate (450 µl of 1% p-nitrophenyl β-D-galactopyranoside, 15 mM NaCl, 0.06% bovine serum albumin (w/v) in 50 mM Na-acetate, pH 5.0) was incubated with 50 µl of enzyme extract for 1 h. The reaction was stopped by adding 1 ml of 0.2 M Na₂CO₃, and released p-nitrophenol groups were measured with a spectrophotometer at 400 nm. The standards contained p-nitrophenol, and controls had 0.2 M Na₂CO₃ added at time 0. Polygalacturonase activity was determined by placing a 100-µl aliquot of acetone powder extract in 900 µl of 1% polygalacturonic acid in a 0.05-M acetate buffer at pH 5 and incubating the tubes at 30°C for 24 h. Dinitrosalicylic acid reagent (DNS) (200 µl) was added to the enzyme and substrate and heated at 100°C for 5 min (Miller, 1959). Reducing groups were determined with a spectrophotometer at 500 nm. Cellulase activity was measured in a similar way, except the substrate was 1% carboxymethyl cellulose (medium viscosity) in 0.05 M acetate buffer, pH 5. Each treatment (harvest date or color group) consisted of three replications, and the experiments were conducted three times within the same harvest.

Statistical analyses were performed with the Statistical Analysis System (SAS Institute, Cary, N.C.). Data were subjected to one-way analyses of variance, and treatment means were compared with Fisher’s protected least significant difference (LSD) test.

Results

Chile peppers increased in fresh weight and volume from 20 to 33 DAF (Fig. 1), and fresh weight and volume significantly decreased in fruit harvested later in the season (89 DAF). Fruit wall firmness increased steadily from 24N at 20 DAF to 36N at 54 DAF (Fig. 2), then declined to 8 N on the last harvest date (103 DAF).

Chlorophyll content followed similar trends in fresh and dry weight samples (Fig. 3). The chlorophyll content decreased significantly from 20 to 33 DAF and then maintained the same level for ≈ 4 weeks. By 60 DAF, chlorophyll content had decreased significantly. At this time, pepper fruit were beginning to show orange and red pigmentation (breaker/turning stage). Green and red fruit harvested on the same day also showed significant differences in chlorophyll content (Table 1). The loss of chloro-
Phytochrome corresponded to changes in the Land a/b color values (Table 1); as chlorophyll content decreased, a : b ratios increased, and L values decreased.

Carbon dioxide production decreased seasonally from 174 to 24.4 ml·kg⁻¹·h⁻¹ (Fig. 4). There was no climacteric rise of CO₂ at the time of color change. The difference in respiration rate also was observed among the fruit harvested on the same day, but of different color (Table 1). Green peppers harvested 63 DAF had significantly higher rates of CO₂ production than red peppers harvested the same day. In contrast, the highest peak of C₂H₄ production (0.20 µl·kg⁻¹·h⁻¹) was at 69 DAF (Fig. 4), when the fruit were completely red but still succulent. A smaller C₂H₄ peak corresponded to the rapid weight and volume gains observed at 33 DAF. Also, red peppers showed significantly higher levels of C₂H₄ production than the other maturity groups (Table 1) harvested on the same day.

Cellulase and polygalacturonase activity were not detected using the DNS assay. ß-galactosidase specific activity increased 7-fold (54 to 69 DAF) when compared to the early harvests (20 to 47 DAF) (Fig. 5). Results were similar when expressed on a fresh weight basis. ß-galactosidase activity remained higher in the red than in the green fruit until dehydration was evident (103 DAF). Fruit from the R/G and R maturity groups harvested on the same day had higher ß-galactosidase activity than the G/R or G groups (Table 1).

Table 1. Color and physiological changes in chile peppers harvested at four stages of maturity.

| Ripeness class | Color (L) | Color (a/b) | Chlorophyll (mg·mg⁻¹ fresh wt) | Ethylene (µl·kg⁻¹·h⁻¹) | CO₂ (ml·kg⁻¹·h⁻¹) | ß-galactosidase (mmol·mg⁻¹·h⁻¹) |
|----------------|-----------|-------------|--------------------------------|------------------------|-------------------|-------------------------------|
| Green          | 42.43     | 0.65        | 28.0 a                          | 0.15 b                 | 79.75 a           | 9.3 c                         |
| Green/red      | 40.11     | 0.34        | 22.12 ab                        | 0.12 b                 | 78.5 a            | 17.9 bc                        |
| Red/green      | 35.93     | 0.72        | 21.3 ab                         | 0.13 b                 | 73.4 ab           | 45.5 a                         |
| Red            | 35.47     | 2.09        | 16.6 b                          | 0.27 a                 | 63.9 b            | 29.1 b                         |

*The color of the fruits in the ripeness classes were: green = 100% green, green/red = 10% to 20% red, red/green = 50% to 60% red, and red = 100% red.

*mmol p-nitrophenyl/mg protein/h

*Mean separation within columns according to Fisher’s LSD (P ≤ 0.05).
SDS-PAGE separation of total proteins revealed increased protein band intensities at 27, 35, and 40 kDa and decreased band intensity at 51 kDa during the season (Fig. 6A). The same bands increased in intensity with maturity of fruit in proteins from fruit harvested on the same day (Fig. 6B).

**Discussion**

New Mexican type peppers were harvested weekly and analyzed for physiological changes related to the maturation and ripening of the fruit. Chile peppers from the first harvest (20 DAF) were noticeably small and undeveloped. Fruit weight and volume increased rapidly in the early harvests from 20 to 33 DAF, and reached a plateau at 40 DAF, when peppers were fully developed and mature-green. Peppers harvested 69 DAF were firm, red, and succulent. At 75 DAF, fruit weight and volume began to decrease as fruit started to desiccate on the plant, a characteristic of the New Mexican type pepper. As fresh weight decreased, fruit firmness also decreased. In bell pepper fruit, weight loss correlated highly with decreased firmness and lower water potential (Lurie et al., 1986). They also found that fruit water potential at harvest was higher for green than red peppers, and firmness decreased as the color change advanced.

Chile pepper respiration rates were highest in immature, green pods and continued to decrease as maturation and ripening proceeded throughout the season. Typically, fruit harvested during an active growth phase have high respiration rates that generally decline as the fruit mature (Kader, 1987). Climacteric-type fruit show a unique ripening pattern, with a burst of respiratory activity and C\textsubscript{2}H\textsubscript{4} production coincident with ripening (Biale, 1964). No respiratory climacteric was observed as chile peppers developed and ripened over the season. However, a CO\textsubscript{2} increase may have occurred between the weekly sampling intervals and, therefore, was not observed. Also, aclimacteric increase in chile peppers may not be evident in freshly harvested fruit. In studies with ‘Honey Dew’ melons (Cucumis melo L.), Pratt et al. (1977) found that full-size melons held in storage exhibited a respiratory climacteric, whereas Miccolis and Salveit (1991) did not observe aclimacteric rise for ‘Honey Dew’ fruit harvested from the field and measured 5 h later. They postulated that the climacteric increase may not occur in freshly harvested melons when compared to stored fruit. Our classification of chile peppers as nonclimacteric agrees with postharvest studies of bell peppers (Lurie et al., 1986; Salveit, 1977) and ‘Changjiao’ hot peppers (Lu et al., 1990), which also have a nonclimacteric ripening pattern.

Ethylene production increased ≈ 5-fold as chile fruit matured and ripened throughout the growing season. A large rise occurred when fruits were harvested 61 DAF, with a peak at 69 DAF. Coincident with peak C\textsubscript{2}H\textsubscript{4} production, fruit firmness decreased, β-
galactosidase activity increased, and fruit changed from green to red. A smaller \( \text{C}_\text{H} \) peak at 33 DAF corresponded with the rapid growth and expansion of young pods. When chile from different maturity groups were harvested on the same day (Table I), \( \text{C}_\text{H} \) production for 100% red fruit was twice as high as that for mature green fruit. Bell peppers responded similarly: \( \text{C}_\text{H} \) increased 2-fold as mature green peppers ripened and turned red (Lurie et al., 1986; Salveit, 1977).

The peak \( \text{C}_\text{H} \) level for chile peppers was 0.27 \( \mu \)l·kg\(^{-1}\)·h\(^{-1}\), compared to 0.70 \( \mu \)l·kg\(^{-1}\)·h\(^{-1}\) for ‘Chooreaehong’ hot peppers (Gross et al., 1986). In general, the \( \text{C}_\text{H} \) rates for peppers are low (0.1 to 1.0 \( \mu \)l·kg\(^{-1}\)·h\(^{-1}\)) when contrasted with tomatoes (\textit{Lycopersicon esculentum} Mill.), which may reach \( \text{C}_\text{H} \) production rates of 10 \( \mu \)l·kg\(^{-1}\)·h\(^{-1}\) at the climacteric peak (Biale, 1964). Ethylene generally has been accepted as the natural ripening hormone based on physiological evidence. Recently, molecular inactivation of \( \text{C}_\text{H} \), biosynthesis has shown conclusively that ethylene controls tomato fruit ripening and many of the biochemical changes associated with it (Oeller, 1991). Although peppers are classified as nonclimacteric, \( \text{C}_\text{H} \) is synthesized at levels sufficient to induce ripening. However, unlike tomatoes, a respiratory rise does not indicate the onset of ripening, and the loss of green pigmentation and ripening may proceed more slowly.

In addition to changes in respiration and \( \text{C}_\text{H} \) production, the fruit ripening process often involves changes in enzymatic activity related to cell wall degradation. Whereas ripening tomatoes show increased levels of PG and cellulase activity (Babbitt et al., 1973), PG and cellulase were undetected for chile peppers under the assay conditions described. \( \beta \)-galactosidase specific activity increased 7-fold in ripening chile peppers, similar to that reported for ‘Chooreaehong’ hot peppers, in which total \( \beta \)-galactosidase activity increased 15-fold, and PG was undetected (Gross et al., 1986).

The exact role of \( \beta \)-galactosidase is unknown, but the loss of galactose residues from cell walls may be a result of this enzyme (Gross and Sams, 1984; Pressey, 1983). \( \beta \)-galactosidase was found to degrade apple galactans and increase as apples matured (Bartley, 1974). Pressey (1983) found three \( \beta \)-galactosidase isozymes in tomato and one of the isozymes hydrolyzed tomato galactan. \( \beta \)-galactosidase was found at high levels in the tomato, and the hydrolytic isozyme increased during fruit ripening. The loss of galactose and arabinose containing cell wall polysaccharides has been associated with fruit ripening in several species (Gross and Sams, 1984). Galactose was the major residue lost in seven species, among them peppers (bell and hot). Thus, the dramatic increase in \( \beta \)-galactosidase activity as chile peppers ripened would be expected.

Distinct protein changes were found between harvest dates (Fig. 6A) and among maturity groups (Fig. 6B), including decreased intensity of a 5 1-kDa protein. A 40-kDa band appeared to increase in intensity as the fruit turned red. At 47 DAF, a 35kDa protein band increased suddenly. The appearance of this band precedes fruit color change from green to red, decreased firmness (Fig. 2), chlorophyll loss (Fig. 3), and \( \text{C}_\text{H} \) increase (Fig. 4), and correlates closely with the beginning of Increased \( \beta \)-galactosidase activity (Fig. 5). Pressey (1983) found three \( \beta \)-galactosidase isozymes in tomato, and one of the isozymes hydrolyzed tomato galactan and increased with fruit ripening. The molecular weight of the three isozymes were found to be 144,000 (I), 62,000 (II), and 71,000 (III) using column chromatography. Proteins increasing with pepper maturity in this study were smaller than 71 kDa; however, denaturation may have affected the original conformation.

Characterization of \( \beta \)-galactosidase in peppers has been limited. Gross et al. (1986) reported the presence of at least four isozymes in hot peppers. Preliminary investigations using a Mono-S chromatography column (Pharmacia, Piscataway, N.J.) has also shown the presence of four isozymes in New Mexican type peppers (Biles, Wall, and Kuehn, unpublished). Further work is needed in purifying pepper \( \beta \)-galactosidase and the 40-, 35-, and 27-kDa proteins to understand their function.

In conclusion, New Mexican type chile peppers appear to be nonclimacteric, although a rapid increase of \( \text{C}_\text{H} \) corresponds with color change. Corresponding with the rise in \( \text{C}_\text{H} \), is a sudden loss of chlorophyll, decrease in firmness, an increase of \( \beta \)-galactosidase activity, and the appearance of a new protein band at 35 kDa. These characteristics require further investigation to determine their role in chile pepper development and ripening. Manipulation of these variables may ultimately lead to enhanced quality and extension of shelf life.

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