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 (C_2H_4) 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 CO_2 was absent. There were two peaks in C_2H_4 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.

The New Mexican type pepper 'New Mexico 6-4' is an integral part of the unique culture of the southwestern United States. In New Mexico, this pepper type is commonly called Chile. Chile peppers are either pungent or nonpungent, green or red, 15 to 18 cm long, and $\approx$ 5 cm wide (Bosland et al., 1988). When harvested green, New Mexican Chile peppers are eaten fresh, canned, or frozen. If allowed to mature on the plant, the peppers turn red and eventually dehydrate. Dried red Chile fruit are ground into Chile powder or paprika.

The growth and lengthening of green Chile pods follows a single sigmoidal curve, similar to the growth pattern for bell pepper fruit (Miller et al., 1979; Pety and Cotter, 1984). No studies have investigated the physiological changes that occur during the maturation and ripening of Chile peppers. Whereas bell peppers are generally classified as nonclimacteric fruits (Lurie et al., 1986; Miller et al., 1979; Salveit, 1977), Korean hot pepper types have been reported as either climacteric for *C. annuum* L. 'Choorahong' (Grosset et al., 1986) or nonclimacteric for *C. annuum* L. 'Changjiao' (Lu et al., 1990). Nonclimacteric fruit do not show the dramatic rise in respiration and ethylene production during ripening that is commonly observed with climacteric fruit (Biale, 1964). The ripening behavior of New Mexican type peppers, with respect to respiration and ethylene production rates, is unknown.

Ripening-related changes in cell wall composition have been documented for many fruit species. In a survey of 17 fruit types, hot peppers had the greatest net loss (56%) of noncellulosic neutral sugars from the cell walls during ripening. Galactose was the major residue lost (Gross and Sams, 1984). β -galactosidase activity increased, whereas polygalacturonase (PG) activity was undetected. Our objectives were to determine the maturation and ripening related changes in New Mexican type peppers. Variables mea-

sured were polygalacturonase (E.C. 3.2.1.15), cellulase (E.C. 3.2.1.4), and β -galactosidase (E.C. 3.2.1.23) activities, CO_2 , and C_2H_4 production rates, chlorophyll content, and fruit firmness.

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 CO_2 . C_2H_4 and CO_2 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 CO_2 was determined using an infrared gas analyzer (Illinois Instruments, McHenry, Ill.) calibrated with a 1.0% CO_2 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 C_2H_4 /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

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Abbreviations: DAF, days after flowering; C_2H_4 , ethylene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DNS, dinitrosalicylic acid.

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 as 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 $\approx$ 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 -40C 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 $\approx$ 30 ml of acetone 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 -20C. Protein was extracted with distilled deionized water (24C) 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 $\times$ g. Extracts used for gel electrophoresis were filtered through a 0.22- μ m filter after centrifugation. Proteins were concentrated through addition of 100 μ l of 100% trichloroacetic acid (TCA) to 900 μ l 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 100C. Total protein (4 μ g/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 μ l 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 μ l of enzyme extract for 1 h. The reaction was stopped by adding 1 ml of 0.2 M Na_2CO_3 , and released p-nitrophenyl groups were measured with a spectrophotometer at 400 nm. The standards contained p-nitrophenyl, and controls had 0.2 M Na_2CO_3 added at time 0. Polygalacturonase activity was determined by placing a 100- μ l aliquot of acetone powder extract in 900 μ l of 1% polygalacturonic acid in a 0.05-M acetate buffer at pH 5 and incubating the tubes at 30C for 24 h. Dinitrosalicylic acid reagent (DNS) (200 μ l) was added to the enzyme and substrate and heated at 100C 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 $\approx$ 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-

Fig. 1. Fruit weight (■) and volume (□) of New Mexican type peppers harvested weekly. Each point represents the mean of measurements taken from 12 fruit at each harvest date. Error bars represent the SE of the mean. $\text{LSD}_{(0.05)}$ values for fruit weight and volume were 6.5 and 12.0, respectively.

Fig. 2. Fruit firmness for New Mexican type chile harvested at weekly intervals throughout the season. Arrows indicate the approximate start of color change from green to red (breaker) and when 25% of the fruit was near dehydration on the plant. Twelve fruit were tested at each harvest date with three measurements taken on each fruit. Error bars represent the SE of the mean. The $LSD_{(0.05)}$ value was 3.4.

Fig. 3. Fresh (■) and dry weight (□) chlorophyll content of New Mexican type chile fruit harvested at weekly intervals throughout the season. The arrow indicates the approximate start of color change from green to red (breaker). Twelve fruit were tested at each harvest date with six disks taken from each fruit. Error bars represent the SE of the mean. The $LSD_{(0.05)}$ values for the fresh and dry weight chlorophyll contents were 0.049 and 0.064, respectively.

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

Ripeness class ²	Color (L)	Color (a/b)	Chlorophyll (mg·mg ⁻¹ fresh wt)	Ethylene (μl·kg ⁻¹ ·h ⁻¹)	CO ₂ (ml·kg ⁻¹ ·h ⁻¹)	β-galactosidase (mmol·mg ⁻¹ ·h ⁻¹) ³
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

²The 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.

³mmol p-nitrophenyl/mg protein/h

^{*}Mean separation within columns according to Fisher's LSD ($P \leq 0.05$).

phyll 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. β-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. β-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 β-galactosidase activity than the G/R or G groups (Table 1).

Fig. 4. Carbon dioxide (□) and ethylene (■) evolution of New Mexican type chile peppers harvested at weekly intervals throughout the season. Four replications of three fruit each were used for each measurement. Error bars represent the SE of the mean. The $LSD_{(0.05)}$ values for CO₂ and ethylene data were 17.5 and 0.098, respectively.

Fig. 5. β -galactosidase activity of New Mexican type peppers at each harvest date through the season. Error bars represent the SE of the mean. The $LSD_{(0.05)}$ for the β -galactosidase data was 2.71.

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_2H_4 production coincident with ripening (Biale, 1964). No respiratory climacteric was observed as chile peppers developed and ripened over the season. However, a CO_2 increase may have occurred between the weekly sampling intervals and, therefore, was not observed. Also, a climacteric 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 a climacteric 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_2H_4 production, fruit firmness decreased, β -

Fig. 6. Denatured protein patterns of New Mexican type peppers harvested throughout the growing season (A) and harvested on the same day (B). The numbers 1 to 11 at the bottom of gel A correspond to peppers harvested 20, 33, 47, 54, 61, 75, and 103 days after flowering, respectively. Abbreviations at the bottom of gel B represent fruit maturity groups: G = 100% green, G/R = 10% to 20% red, R/G = 50% to 60% red, and R = 100% red. BioRad low molecular weight standards were loaded in the far left lane on each gel.

galactosidase activity increased, and fruit changed from green to red. A smaller C_2H_4 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), C_2H_4 production for 100% red fruit was twice as high as that for mature green fruit. Bell peppers responded similarly; C_2H_4 increased 2-fold as mature green peppers ripened and turned red (Lurie et al., 1986; Salveit, 1977).

The peak C_2H_4 level for chile peppers was $0.27 \mu\text{l}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, compared to $0.70 \mu\text{l}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ for 'Chooreahong' hot peppers (Gross et al., 1986). In general, the C_2H_4 rates for peppers are low (0.1 to $1.0 \mu\text{l}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) when contrasted with tomatoes (*Lycopersicon esculentum* Mill.), which may reach C_2H_4 production rates of $10 \mu\text{l}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ at the climacteric peak (Biale, 1964). Ethylene generally has been accepted as the natural ripening hormone based on physiological evidence. Recently, molecular inactivation of C_2H_4 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, C_2H_4 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 C_2H_4 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. β -galactosidase specific activity increased 7-fold in ripening chile peppers, similar to that reported for 'Chooreahong' hot peppers, in which total β -galactosidase activity increased 15-fold, and PG was undetected (Gross et al., 1986).

The exact role of β -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). β -galactosidase was found to degrade apple galactans and increase as apples matured (Bartley, 1974). Pressey (1983) found three β -galactosidase isozymes in tomato and one of the isozymes hydrolyzed tomato galactan. β -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 β -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 kDa protein. A 40-kDa band appeared to increase in intensity as the fruit turned red. At 47 DAF, a 35 kDa 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 C_2H_4 increase (Fig. 4), and correlates closely with the beginning of increased β -galactosidase activity (Fig. 5). Pressey (1983) found three β -galactosidase isozymes in tomato, and one of the isozymes hydrolyzed tomato galactan and increased with fruit ripening. The molecular weight of the three isoforms 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 β -galactosidase in peppers has been lim-

ited. 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 β -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 C_2H_4 corresponds with color change. Corresponding with the rise in C_2H_4 is a sudden loss of chlorophyll, decrease in firmness, an increase of β -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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