Plant Physiol. (1978) 61, 416–419

Cellulase Activity and Fruit Softening in Avocado[1]

Received for publication May 24, 1977 and in revised form October 18, 1977

EDNA PESIS, YORAM FUCHS, AND GIORA ZAUBERMAN
Division of Fruit and Vegetable Storage, Institute for Technology and Storage of Agricultural Products, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan, Israel

ABSTRACT

Cellulase activity in detached avocado (*Persea americana* Mill.) fruits was found to be directly correlated with ripening processes such as climacteric rise of respiration, ethylene evolution, and softening. This activity in the pericarp could be induced by ethylene treatment, and the more mature the fruit—the faster and the greater was the response. Only a very low cellulase activity could be detected in hard avocado fruit right after harvest. Cellulase activity was highest at the distal end of the fruit, lower in the midsection, and lowest at the proximal end. The enzyme is heat-labile and appeared to have activity of an endocellulase nature mainly. Electron micrographs of cell walls from hard and soft fruits are presented.

The most obvious feature of avocado fruit ripening is softening, and the most common physiological parameters for determining avocado ripening are ethylene evolution and respiration rate (4). It is generally believed that softening of fruits during ripening is related to alteration in the pectic substances through action of pectic enzymes (4). A clear correlation between the activities of polygalacturonase and pectin methylesterase and fruit softening in avocado has been shown (3, 23). Also, differences in polygalacturonase activity (17) and in ethylene production and respiration (21) between the different parts of avocado fruit were reported. It has been suggested that cellulase, in addition to pectic enzymes, might contribute to softening of tomato fruits (7, 13, 15) and of dates (12). Cellulose was reported to be the main constituent of young avocado fruit cell walls (19). Cellulase activity in freeze-injured avocado fruits has been reported (10). The ultrastructure of plant cell walls has been described in detail (2, 14), and some ultrastructural aspects of avocado fruit have been reported (19).

It was of interest to study what might be the involvement of cellulase during normal and ethylene-induced ripening processes in avocado. Therefore, changes in cellulase activity were investigated in fruits which were harvested at various stages of maturity and treated with ethylene. Some characteristics of the partially purified enzyme and some ultrastructural changes in the cell walls were studied.

MATERIALS AND METHODS

Plant Material. Avocado (*Persea americana* Mill.) fruits of the 'Fuerte' cultivar were harvested periodically, starting in June with very young fruit (62 g and 1.6% oil content), and ending in December with completely mature fruit (313 g and 14.9% oil content). These fruits were stored at 20 C. Starting on the day of harvest, ethylene was applied for 48 hr in a flow system, at a concentration of 50 μl/l; pure air was supplied to the controls.

Acetone powder was prepared from fruit pulp; 1 part of pulp and 8 parts (w/v) of acetone at −20 C were homogenized in a blender. After vacuum filtration the powder was blended a few more times with cold acetone until a bright colorless powder was obtained. The powder was dried at room temperature and then milled in a coffee mill and stored at −20 C until extraction.

Tissue for electron microscopy was fixed from mature fruits (17.4% oil content, 11.2 kg firmness) on the day of harvest and after 11 days of storage in the dark at 14 C. These fruits were also used for the studies summarized in Figure 2.

Electron Microscopy. Tissue (1 × 1 mm) from the "pale green" zone of the pericarp was fixed at room temperature for 2 hr with 4% glutaraldehyde and 0.1 M phosphate buffer (pH 7.5). The tissue was rinsed in buffer and postfixed in 2% OsO_4 in the same buffer for 1 hr at room temperature. It was dehydrated in an alcohol series followed by propylene oxide and embedded in Epon 812. Silver-gray sections were cut, mounted on copper grids, stained in uranyl acetate-lead citrate, and viewed in a Jeol electron microscope (JEM-T7).

Enzyme Extraction and Partial Purification. Acetone powder (400 mg) was extracted with 10 ml of 0.02 M phosphate buffer at pH 6.6 for 1 hr at 1 C. Also, extractions were tested with 1 M NaCl added to the extracting buffer. The slurry was centrifuged for 20 min at 30,000*g* at 1 C. The supernatant was decanted and then filtered through a Millipore filter (1.2 μ). One ml of the filtrate was applied on a Sephadex G-25 column (1.7 × 18 cm) and eluted with 0.02 M phosphate buffer at pH 6.6. Fractions of 5 ml were collected and assayed for cellulase activity, and the optical density at 280 nm was measured for protein content determination, using a BSA calibration curve (Fig. 1). The fractions containing the main cellulase activity were combined to give the "enzyme solution."

Cellulase Assay. Enzyme solution (5 ml) and 10 ml of substrate (1% CMC,[2] BDH), dissolved in 0.1 M acetate buffer at pH 5.5 containing 0.1% synthomycetin and 0.1% cycloheximide, were incubated at 30 C in an Ostwald viscometer head (Volac No. 150), and readings were recorded at 10-min intervals for 60 min. The optimal pH was found to be between 4.5 and 6.0. A unit of cellulase activity was defined as the amount of solution which causes a 1% loss in relative viscosity/hr of the reaction mixture. A reaction mixture containing 5 ml of boiled enzyme solution was used as a blank.

Reducing Groups. In some of the experiments Sumners' reagent (20) was used to measure changes in reducing groups as an indication of cellulase activity. For this determination, 5 ml of enzyme solution was incubated with 10 ml of the substrate (CMC as above) at 30 C, and 1 ml of this reaction mixture was boiled 5 min with 2 ml of Sumners' reagent; the reduction product was determined colorimetrically at 550 nm.

Fruit Firmness. Firmness was determined, without removing the epidermis, by a Chatillon pressure tester, using the conical tip, in which the number of kg force to penetrate the pulp is directly correlated to firmness. At about 3.5 kg the beginning of softening could be felt by hand.

[1] Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. 1977 Series, No. 152-E.

[2] Abbreviation: CMC: carboxymethyl cellulose.

Ethylene and CO_2 were determined by gas chromatography (9), and oil content was determined using a refractive index method (11). Data represent at least six experiments which were carried out during two seasons; either averages or representative curves are presented.

RESULTS

Very low cellulase activity was detected on the day of harvest in pericarp from avocado fruits which were harvested at various stages of development. Activity was noticeable only when the fruit began to go through its climacteric rise in respiration and to produce ethylene, but before softening was evident (Fig. 2). No significant differences in final cellulase activity levels were observed between young immature and mature fruits. However, cellulase activity and ripening, in general, became evident more rapidly after harvest in mature fruit than in young fruit. Maximal cellulase activity was attained when the fruit was completely soft (Fig. 2). Cellulase activity during ripening was highest in the distal end, lower in the midsection, and lowest in the proximal end (Fig. 3), while the firmness of these different parts of the fruit was inversely correlated to their cellulase activity. In completely soft fruit no significant differences in cellulase activity or firmness, could be observed among the different parts of the fruit.

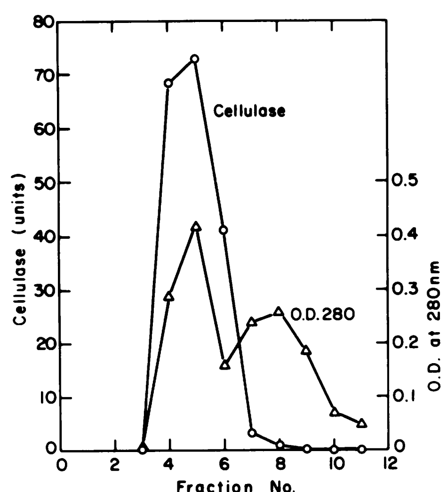

FIG. 1. Initial purification of avocado cellulase by gel filtration.

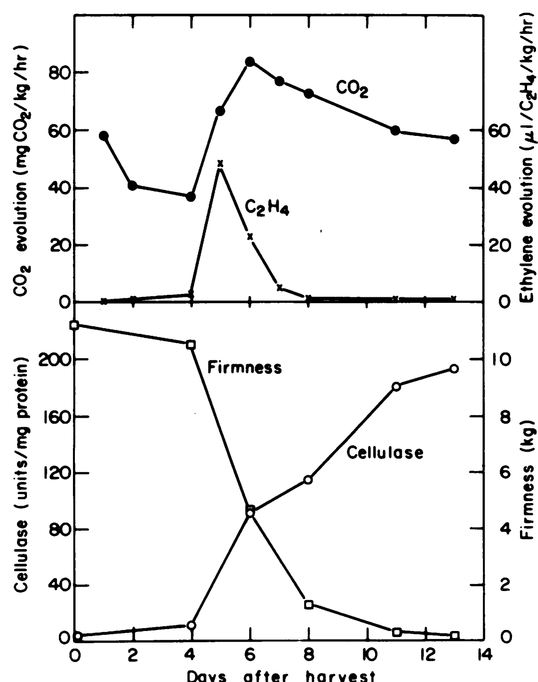

FIG. 2. Changes in cellulase activity, firmness, respiration rate, and ethylene evolution in mature avocado fruit. Cellulase activity is presented as specific activity (units/mg of protein) in the enzyme solution. The fruit was stored at 14 C after harvest.

In Figure 4 the differences in the structure of cell walls from hard and soft fruits are demonstrated. In the hard fruit (Fig. 4, A and B) the middle lamella is obvious and the fibrils are packed tightly in order on both sides of the middle lamella. In soft fruit (Fig. 4, C and D) no middle lamella can be observed, many of the fibrils are missing, and many diagonal fibrils can be seen.

Ethylene treatment had no effect on cellulase activity (measured 48 hr after harvest) in very young fruits harvested in June or July (Fig. 5); later in the season, the more mature the fruit—the higher was the cellulase activity and the more it responded to ethylene.

There was a lag in the increase in the reducing power of the enzymic reaction mixture as compared with the decrease in viscosity (Fig. 6). Addition of 1 M NaCl to the extracting buffer did not affect the cellulase activity of the extract. Incubating the enzyme solution for 0.5 or 2 hr at 30 C before the assay did not affect its activity; incubation for the same length of time at 40, 50, and 60 C inactivated the enzyme, and the higher the temperature and the longer the time—the greater was the inactivation.

DISCUSSION

Since cellulase activity in the avocado fruit increased during ripening (Fig. 2), it is possible that the enzyme has a role in ripening processes, perhaps in conjunction with polygalacturonase (23). It has been suggested (7) that cellulase may act in tomato fruit in conjunction with pectic enzymes to cause softening. The fact that the increase in cellulase activity matches the changes in the ripening, such as increase in respiration rate and ethylene evolution (Fig. 2), is additional supporting evidence for the involvement of cellulase in avocado softening.

In our studies with 'Fuerte' fruits, softening and cellulase activity always occurred earliest at the distal end of the fruit (Fig. 3), as has been previously reported for polygalacturonase activity in this variety (17). In their studies with the 'Hass' variety, Tingwa and Young (21) reported that ethylene production and softening of the fruit were always detected earlier in the stem end than in other parts of the fruit. It seems that there are differences in the sequence of ripening, within the fruit, between the two varieties.

One would expect that as a result of the action of pectic and cellulolytic enzymes, pectins and cellulose in the fruit would be hydrolyzed; indeed, it can be observed that the middle lamella and the orderly tight fibrils which are evident in hard fruit (Fig. 4, A and B) are dissoluted and sparse, respectively, in soft fruit

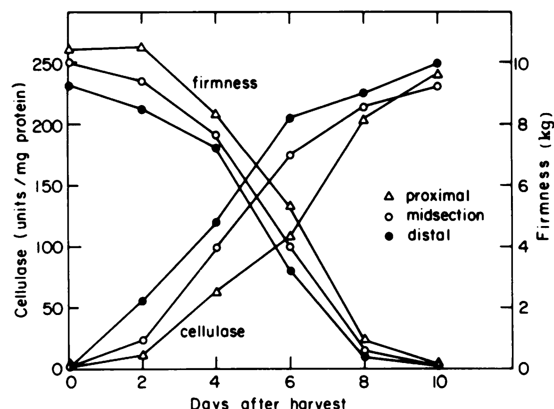

FIG. 3. Changes in cellulase activity and firmness in different parts of avocado fruit during ripening. The fruits were sampled during ripening at 20 C.

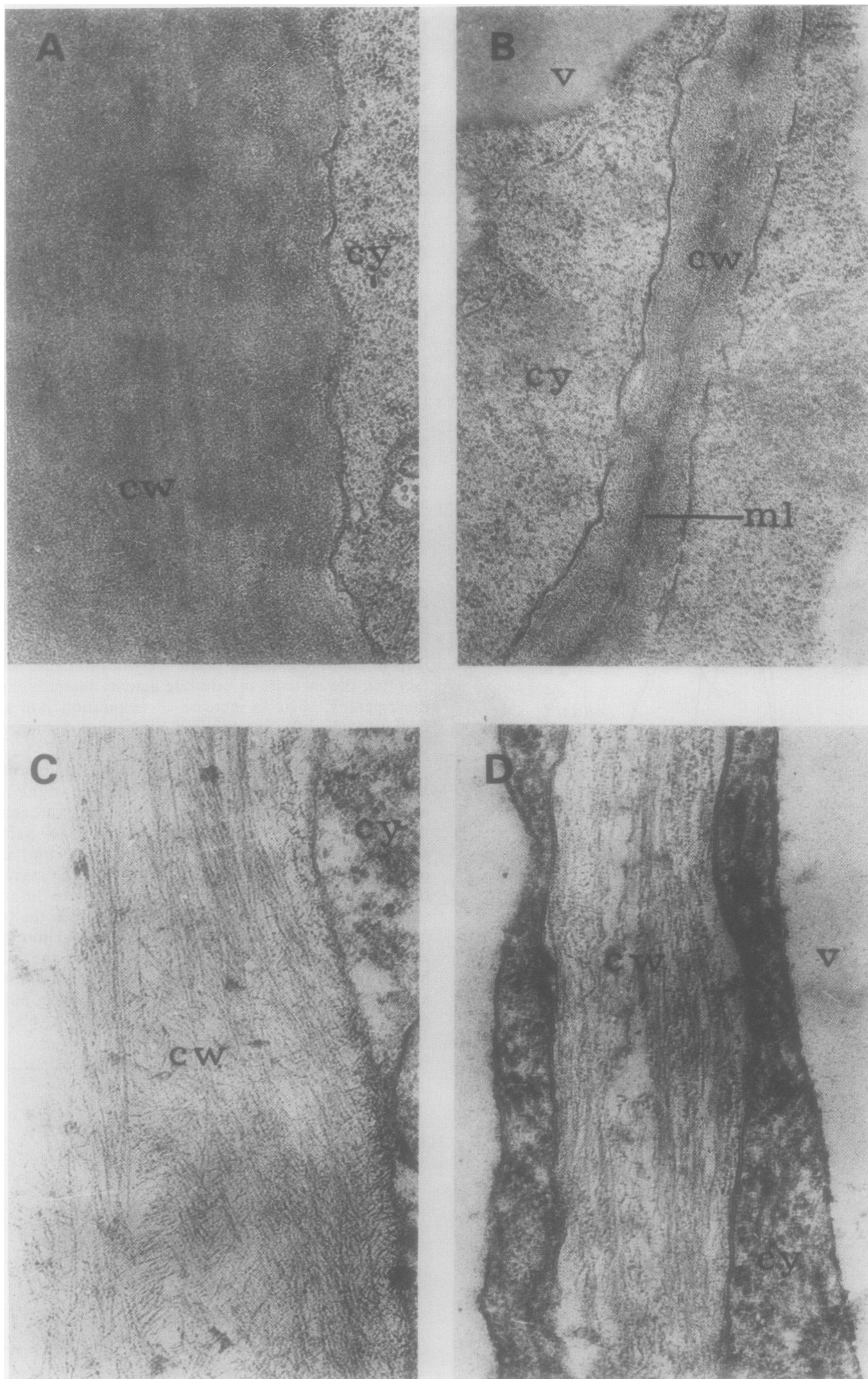

FIG. 4. Electron micrographs of avocado fruit cell walls prepared from: A: hard (firm) fruit (× 29,400); B: hard (firm) fruit (× 29,400); C: soft fruit (× 50,400); D: soft fruit (× 70,000). ml: middle lamella; cw: cell wall; cy: cytoplasm; v: vacuole.

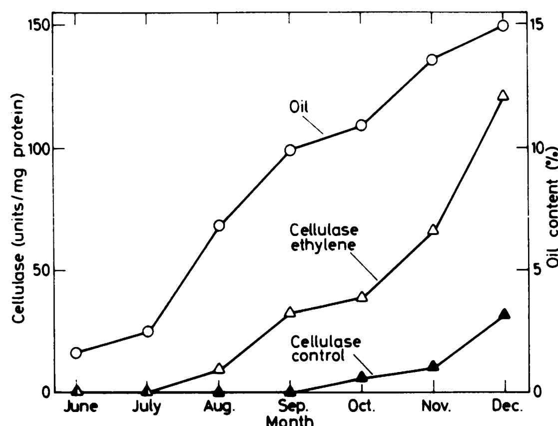

FIG. 5. Effect of postharvest ethylene treatment on cellulase activity at different harvest dates.

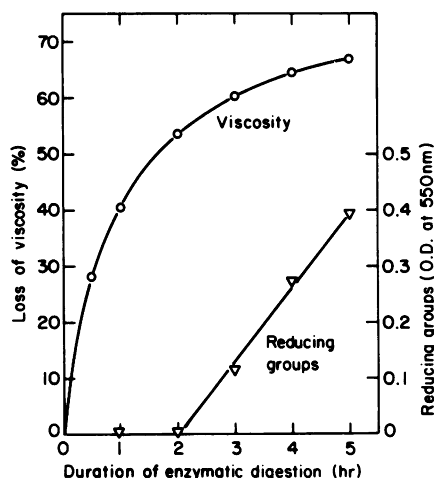

FIG. 6. Cellulase activity of avocado pulp extracts detected by two different methods. Per cent loss of viscosity and the increase in reducing groups of the reaction mixture were recorded for 5 hr. Both methods were employed under the same conditions.

cell walls (Fig. 4, C and D). The cell walls of avocado fruit fit the description of cell wall structure in other reports (2, 14). The diagonal lines which can be observed in cell walls of soft fruits may be hemicellulose structures similar to those described previously (2, 14).

The lag in the rise in reducing groups relative to the rate of loss of viscosity of the enzymic reaction mixture (Fig. 6) may indicate that avocado cellulase is acting as an endocellulase, when CMC is the substrate. Also, cellulase activity in various citrus organs and in other plants was shown to be mainly of an endocellulase nature (5, 18). Evidence based on differential salt extractions in some other plants (5, 8, 15) suggests that more cellulase activity was extracted while increasing the salt concentration in the buffer, and that more than one molecular form of cellulase is involved in the diverse physiological plant processes. In our study, buffer containing 1 M NaCl did not extract (from acetone powder) more cellulase activity than the buffer itself. Also, in tomatoes it has been reported (7) that the addition of salt did not improve the activity in extracts from fresh tissue. Cellulolytic activity in avocado is heat-labile as has been reported for pea cellulase (6).

The more mature the fruit, the greater effect the ethylene treatment had on the cellulolytic activity in it (Fig. 5). It has also been reported that the effect of ethylene on respiration rate of avocado increased with its advancing maturity (22). It has been suggested that there is a hormonal regulation of the development (8, 16) and the secretion (1) of cellulase isoenzymes in some plant tissues. It is suggested here that cellulase, in addition to the pectic enzymes, plays an important role in avocado fruit softening and it appears that ethylene controls its activity.

Acknowledgment—We thank E. Zamski for his help with the electron microscopy studies.

LITERATURE CITED

1. ABELES FB 1971 Abscission: control of cellulase secretion by ethylene. Planta 97: 87-91
2. ALBERSHEIM P 1975 The wall of growing plant cells. Sci Am 232: 81-95
3. BARMORE CR, AH ROUSE 1976 Pectinesterase activity in controlled atmosphere stored avocado. J Am Soc Hort Sci 101: 294-296
4. BIALE JB, RE YOUNG 1971 The avocado pear. *In* AC Hulme, ed, The Biochemistry of Fruits and Their Products, Vol 2. Academic Press, London, pp 1-63
5. BYRNE H, NV CHRISTOU, DPS VERMA, GA MACLACHLAN 1975 Purification and characterization of two cellulases from auxin-treated pea epicotyls. J Biol Chem 250: 1012-1018
6. DAVIS E, GA MACLACHLAN 1969 Generation of cellulase activity during protein synthesis by pea microsomes *in vitro*. Arch Biochem Biophys 129: 581-587
7. DICKINSON DB, JP MCCOLLUM 1964 Cellulase in tomato fruits. Nature 203: 525-526
8. FERRARI TE, PG ARNISON 1974 Extraction and partial characterization of cellulases from expanding pea epicotyls. Plant Physiol 54: 487-493
9. FUCHS Y, M LIEBERMAN 1969 Effect of kinetin, IAA and gibberellin on ethylene production and their interactions in growth of seedlings. Plant Physiol 43: 2029-2036
10. FUCHS Y, G ZAUBERMAN, U YANKO 1975 Freeze injuries in avocado fruit. HortScience 10: 64-65
11. GAZIT, S, R SPODHEIM 1970 Determination of picking date. *In* The Division of Subtropical Horticulture. The Volcani Institute of Agricultural Research, Bet Dagan, pp 83-85
12. HASEGAWA S, DC SMOLENSKY 1971 Cellulase in dates and its role in fruit softening. J Food Sci 33: 588-591
13. HOBSON GE 1968 Cellulase activity during the ripening of tomato fruit. J Food Sci 33: 588-591
14. LAMPORT DTA 1970 Cell wall metabolism. Annu Rev Plant Physiol 21: 235-270
15. PHARR DM, DB DICKINSON 1973 Partial characterization of Cx cellulase and cellobiase from ripening tomato fruits. Plant Physiol 51: 577-583
16. REID PD, FT LEW, LN LEWIS 1971 Cellulase isozymes in *Phaseolus vulgaris* abscission zones. Plant Physiol 47: 5-42
17. REYMOND D, J PHAFF 1965 Purification and certain properties of avocado polygalacturonase. J Food Sci 30: 266-273
18. RIOV J 1974 A polygalacturonase from citrus leaf explants: role in abscission. Plant Physiol 53: 312-316
19. SCOTT FM, BG BYSTROM, F BOWLER 1963 *Persea americana*, meso-carp cell structure, light and electron microscope study. Bot Gaz 124: 423-428
20. SUMNER JB 1924 The estimation of sugar in diabetic urine, using dinitrosalicylic acid. J Biol Chem 62: 287-290
21. TINGWA PO, RE YOUNG 1975 Studies on the inhibition of ripening in attached avocado (*Persea americana* Mill.) fruits. J Am Soc Hort Sci 100: 447-449
22. ZAUBERMAN G, Y FUCHS 1973 Effect of ethylene on respiration rate and softening of avocado fruit at various stages of development. IIF-IIR-Commission C2-Jerusalem, 1973 pp 107-109
23. ZAUBERMAN G, M SCHIFFMANN-NADEL 1971 Pectin methylesterase and polygalacturonase in avocado fruit at various stages of development. Plant Physiol 49: 864-865