Project Number: 86-I10
Project Title: Ethylene and Almond Development
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ANNUAL REPORT
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PROJECT NO: 86-I10: TREE AND CROP RESEARCH, ALMOND DEVELOPMENT

OBJECTIVES

1) Assess the role of increasing levels of the gaseous hormone ethylene in flower pistils after pollination on: embryo sac development, successful fertilization and fruit set.
2) Define field practices which might contribute to an efficient application of the early harvest concept. Because small scale trials have indicated that almonds will respond favorably to ethylene application it is necessary to expand testing to an orchard sale.
3) Conclude research (by Kitren Weis) which further defines the cellular processes tht control hullsplit.

INTERPRETIVE SUMMARY

The gaseous hormone ethylene is produced by many plants at various times in their development. Ethylene serves to regulate plant behavior when present in or around the plant, often acting in concert with other plant hormones. Project 86-I10 was designed to test whether the production of ethylene by almond flowers and/or fruits plays a role in regulating fruit set and/or nut maturation. A large portion of the work performed this year was done to confirm results of work done in 1983, 1984 and 1985.

1. Work done in Spring 1983-1985 showed a clear correlation between successful pollination of 'Nonpareil' almond flowers and the production of ethylene by these flowers. In Spring 1986 cross-, self- and nonpollinated flowers were sprayed with AOA (aminooxyacetic acid), an inhibitor of ethylene synthesis. Sprays were applied in order to prevent ethylene production and thus see what effect an absence of ethylene would have on fruit set. The ethylene-releasing chemical, ethephon, was also used as a spray treatment in combination with the above three pollination treatments.

Interactions between ethylene and auxin, a second plant hormone, have been implicated in fruit set/drop phenomena in various species. The possibility of interactions of this kind in almond was investigated in Spring 1986 through the use of various anti-auxin sprays: CMPA (2-(p-chloro-phenoxy)-2-methyl propionic acid), 2-NAA (2-naphthalene acetic acid) and TCA (trans-cinnamic acid). In addition, the synthetic auxin, NAA (naphthalene acetic acid), was applied to flowers of the 3 pollination treatments. Gibberellic acid (GA3) was used in spray treatments as a third plant hormone which has been found to stimulate parthenocarpy in varous fruits, whether applied to the plant, or found occurring naturally. In addition to chemical treatments, mechanical stimulus was used on nonpollinated flowers by piercing styles with a fine needle to simulate pollen tube growth.

In vivo production of ethylene by pistils treated with ethephon or AOA was measured by gas chromatography and was compared to pollination treatment controls. Fixed ovules of all treatment groups were either sectioned or treated with cell wall-degrading enzymes for embryo sac isolation, after which embryo sacs were evaluated for degree of development and length (indicators of developmental progress).

Elevated levels of ethylene production were measured in pistils which had been cross-pollinated AOA applications depressed levels of ethylene in cross-pollinated flowers to the range seen in nonpollinated controls. While AOA-treated pistils had embryo sacs that were generally less well-developed than those of similarly pollinated controls, the general trend still held in that cross-pollinated, AOA-treated embryo sacs were longer than those in self-pollinated or nonpollinated AOA-sprayed flowers. Ethephon application appeared to stimulate embryo sac development in all pollination treatments. Average embryo sac lengths were always greater in cross-pollinated pistils, regardless of chemical or mechanical treatment. Thus, while AOA appeared to have reduced ethylene production in cross-pollinated flowers, fruit set was not reduced. Anti-auxin-sprayed pistils did not show significantly different embryo sac development compared to auxin-sprayed pistils. Treatments with all chemicals other than AOA resulted in embryo sac lengths much greater than equivalently-pollinated controls. Results suggest that an enhancement of ethylene production by flowers following cross-pollination is not essential for fruit set, and that all of the chemical or mechanical treatments seem to substitute for pollination, at least to some extent, in relation to pollen sac development.

2. A focus of this year's efforts was to definitively assess field applications of ethylene to hasten and improve harvest removal. Rates and timings for the ethylene applications made were based on the results of studies done during the last few years. Relatively full-scale field trials of ethephon were made in Summer 1986 employing doses higher than those previously used. All applications, except one, were made at concentrations ranging from 100 to 200 ppm of ethylene. One single-tree application at 300 ppm was made by backpack sprayer and resulted in phytotoxic symptoms (gumming, leaf drying). Single-tree applications of varying ethylene concentrations made with a backpack sprayer were initiated on July 18, two weeks prior to hullsplit and ended once split began for a total of three sprays per tree at weekly intervals. A speed sprayer application of 200 ppm ethephon to six trees was made at hullsplit. None of the applications made by either backpack or speed sprayer hastened hullsplit/harvest or improved harvest removal when compared to water-sprayed controls and we must conclude that the use of ethephon for the purpose of early harvest in almonds is not practical.

3. Hullsplit presumably involves cell separation process similar to those seen in almond fruit drop. Samples of almond hull were taken throughout the maturation period (April-July, 1985 and 1986) for investigation of the cellular and biochemical processes leading up to hullsplit. Biochemical tests for degradative enzymes involved in ripening of many fruits indicated that polygalacturonase, an enzyme that digests pectin -- an intercellular "glue", may be a controlling factor in hullsplit. Histochemistry of almond hull samples confirmed these findings. 1985 and 1986 results gave distinct evidence that almond fruits carry out a limited self-digestion prior to hullsplit. Because the biochemistry of fruit ripening appears to be similar to that of hullsplit and fruit ripening is clearly ethylene-regulated, we feel that hullsplit, too, may be ethylene-controlled. When almond clusters were gassed with ethylene, polygalacturonase levels and histochemical changes in the cells of the hullsplit (dehiscence) zone mimicked the anatomical signs observed in naturally-splitting almonds.

EXPERIMENTAL PROCEDURES

1. Methods for pollination treatments and gas analysis were similar to those used in 1983-1985, with the addition of ethephon and three antiauxins: CMPA, TCA and 2-NAA. Sprays of growth regulators or inhibitors were applied daily for a 7-10 day period beginning one day after pollination, which was done from February 13-15. Handsprays were applied to runoff.

Hormones

a. ethephon (eth): 10 ppm
b. gibberellic acid (GA3): 100, 250, 500 ppm
c. 1-naphthalene acetic acid (1-NAA): 0.5 mM

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a. aminooxyacetic acid (AOA): 10 mM
b. 2-napthalene acetic acid (2-NAA): 0.5 mM
c. 2-(p-chlorophenoxy)-2-methyl propionic acid (CMPA): 0.5 mM
d. trans-cinnamic acid (TCA): 0.5 mM

A mechanical stimulus was employed by piercing the styles of nonpollinated flowers to simulate pollen tube growth. Ovules were either sectioned or treated with cell wall-degrading enzymes for embryo sac isolation, viewed anatomically and evaluated for embryo sac development.

2. Weekly spray applications of ethephon at levels of 100 to 200 ppm ethephon were made as whole-tree sprays on developing 'Nonpareil' almond fruits with a back pack sprayer. Applications were begun two weeks before hullsplit on July 18 and continued until hullsplit began for a

total of three sprays per tree. This method was designed to increase sample size from several clusters of nuts per treatment to entire tree load. In addition, the rates used in 1986, which included a single-tree application of 300 ppm ethephon, tested the upper range of the almond's sensitivity to this compound.

A single speed sprayer application of either water or 200 ppm ethephon to two groups of six trees at hullsplit was intended to simulate actual orchard conditions. All treated trees were shaken at harvest and percent drop was determined by counting nuts removed as a percentage of the total crop for individual trees.

All spray treatments were compared to water-sprayed control trees.

3. 'Nonpareil' almonds were sampled approximately once weekly during 1985 beginning April 18 and ending July 18. In 1986, samples were collected once weekly from June 19 to July 7 and then twice weekly until July 17, at hullsplit. Tissue samples from almond hulls were separated into two groups, dehiscence zone and non-DZ materials, which were extracted for cell wall-hydrolyzing enzymes. Enzyme assays of DZ and control tissue samples were compared throughout the maturation process for presence and quantities of key enzymes.

Samples for anatomical and cytochemical studies of the dehiscence zone were collected at 10 days after anthesis, (February 25, 1986), and then weekly from April 18, 1986 until July 18. A variety of cytochemical and histological stains were used to characterize the cell wall constituents of DZ cells and changes in these with development and maturation of the almond fruit.

RESULTS

1. Data from 1983 and 1984 showed that ethylene production by excised almond pistils increases with application of compatible pollen and that this increase parallels embryo sac development (Figure 1). Pollinated and nonpollinated flowers that had been sprayed with AOA in 1985 and 1986 produced low levels of ethylene that were similar to those of non-pollinated, unsprayed controls (Figure 2). Embryo sac development in AOA-sprayed flowers was greater in cross-pollinated pistils than in nonpollinated pistils. Unlike the unsprayed pollination treatments of 1983 and 1984, however, no ovules aborted and all embryo sacs achieved at least the 7-celled, 8-nucleate condition. Ethephon appeared to stimulate embryo sac development in all pollination treatments, and like all other chemical or mechanical treatments, seemed to substitute for pollination to some extent (Figure 3).

2. Data from 1983 and 1984 indicated that hand-sprays of ethephon at low levels (5 and 10 ppm) may enhance the rate of hullsplit in almond and that endogenous ethylene probably is a natural regulator of almond fruit dehiscence and abscission. Similar experiments performed in 1985, however, showed only a moderate effect on the rate of maturation when ethylene-releasing compounds (ethephon and CGA-15281) were used at 20

ppm (Figure 4). When entire young 'Nonpareil' trees in Durham were sprayed with these same compounds at the same concentrations, no effect on rate of maturation was seen (Figure 5). However, when clusters of almond fruits were enclosed and gassed with 3-4 ppm ethylene, maturation and hullsplit rate were clearly enhanced (Figure 6).

Large scale spray trials in 1986 did not result in enhanced maturation rates with any of the treatments used (Figure 7). At the highest level of ethephon used (300 ppm), mild phytotoxic effects were found. Our results lead us to conclude that ethephon can not be practically applied to almond trees to obtain early harvest without accompanying deleterious symptoms.

3. Cytochemistry and anatomy of the dehiscence zone (DZ) of the almond hull were characterized in 1985 and 1986. The DZ is preformed as the suture in the flower bud. At anthesis the DZ is two cell-layers wide and the cells are already differentiated as unique layers of the fruit wall. During growth and developoment of the kernel and hull, the DZ expands in the endocarp region (that portion of the hull which will become the stony shell) to form a distinct region 4-5 cells wide. DZ cells in this region form thick stony walls and ultimately resemble other cells of the shell. The DZ in the mesocarp does not undergo this process of lignification and remains narrow with relatively thin-walled cells. Throughout the DZ, heavy layers of pectin are formed between the cells. The appearance and disappearance of pectins in various areas of the DZ were measured by microspectrophotometry and expressed as "% mean intensity" (Figure 8). A sharp decline in this value in the outer layers of the hull correlated with a relatively large amount of polygalacturonase detected in these tissues shortly before hullsplit. Levels of pectins were considerably lower in the outer layers of the hull (exocarp and mesocarp) of fruits that were gassed with 3-4 ppm ethylene in mid-June than in those of non-gassed fruits collected on the same day. The carbohydrate makeup of that portion of the cell wall within the DZ which is active in hullsplit seems to be largely pectinaceous in nature, as indicated by the stain and enzyme treatments outlined in Figure 9. Those stains which gave a positive result in that they stained portions of the wall in question are regularly used as indicators of the presence of pectins.

Anatomical and cytochemical studies of cell wall changes at the level of the electron microscope are currently being made with completion anticipated in Spring, 1987. These results will be compared to those acquired at the light microscope level.

DISCUSSION

1. Ethylene production by flowers has often been correlated with pollination, petal senescence, and flower drop. Ethylene emitted shortly after pollination seems to be responsible for many post-pollination effects. Because more ethylene is produced following cross-pollination than in nonpollinated flowers and because relatively high levels continue to be produced several days after pollination, we previously thought that the later-produced (5-9 days post-pollination)

ethylene contributes to fruit set and development rather than floral degeneration. 1985 and 1986 results, showing a reduction of ethylene production caused by AOA, but continued embryo sac development, seem to indicate that the production of ethylene, in this case, is not required for control of embryo sac development. An enhancement of embryo sac development in nonpollinated flowers following applications of NAA, GA, anti-auxins, ethephon or piercing suggests that the reproductive process and fruit set in almond may be influenced by more than one growth hormone.

2. The data shown in Figure 6 indicated that the almond fruit is responsive to ethylene gas at fairly low levels. Spray data from 1983-1985 show inconsistencies that may be due to year-to-year weather and orchard variations, differences among treatment starting dates relative to the onset of hullsplit, and inadequate sample size. While 1985 spray experiments were designed to factor out as many of these inconsistencies as possible, our results did not conclusively demonstrate that ethylene controls dehiscence and abscission. Our results of 1986 field trials forced us to conclude that current field application methods for ethylene are impractical for use on almonds in spite of the fact that these fruits will respond to low concentrations of the hormone when the gas is provided to enclosed clusters over a period of a few days prior to hullsplit.

3. Anatomical investigations of the dehiscence zone in maturing almond fruits have provided a clearer picture of the nature of hullsplit in almond. This is an area in plant research which has had little attention in the past and results have led us to a better understanding of the processes involved. A specialized zone of cells develops sensitivity to endogenous controls during maturation. Specialized cell walls apparently break down in response to digestive enzymes that are in highest quantity in the dehiscence zone. Similar enzymes have been shown to be essential in abscission phenomena in other plant parts and in ripening of other fruits. Ethylene-treated almond fruits exhibited cellular changes in dehiscence zone cells which mimicked the cellular changes seen in DZ cells of ready-to-split, mature almond fruits. These results again imply an important role for the plant hormone, ethylene, in almond fruit maturation (dehiscence and abscission).

Because studies to date show no conclusive benefit from ethylene applications, this project will not seek Almond Board funding in 1987; however, some field work is planned next season in which ethylene applications will be initiated somewhat earlier.

NOTE: Graduate student Kitren Weis is completing her studies on the role that ethylene plays in embryo sac development, fruitset and enzymatic control of hullsplit.

PUBLICATIONS/REPORTS

Weis, K.G. and J.M. Labavitch. 1985. Control of almond fruit abscission. Proceedings of the 66th Annual Meeting of the Pacific Division of the American Academy of Science.

Weis, K.G. and V.S. Polito. 1985. Postpollination events in almond flowers: plant growth substances as related to embryo sac development. Acta Horticulturae; Proceedings of the 5th International Symposium on Growth Regulators in Fruit Production. Acta Horticulturae 179. Growth Regulators, p. 379-380.

Weis, K.G., J.M. Labavitch and V.S. Polito. 1986. Physiology and histochemistry of almond fruit dehiscence. XXIInd International Horticultural Congress.

Weis, K.G. and V.S. Polito. 1985. Embryo sac development in almond as related to plant growth substances. XXIInd International Horticultural Congress.

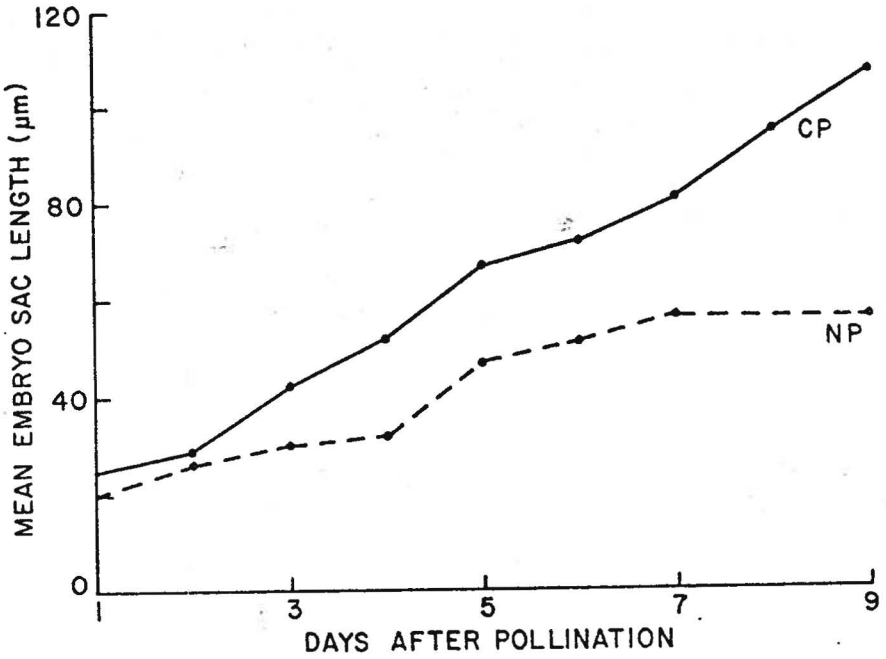

FIG. 1 Embryo sac length in pollinated and nonpollinated almond flowers.

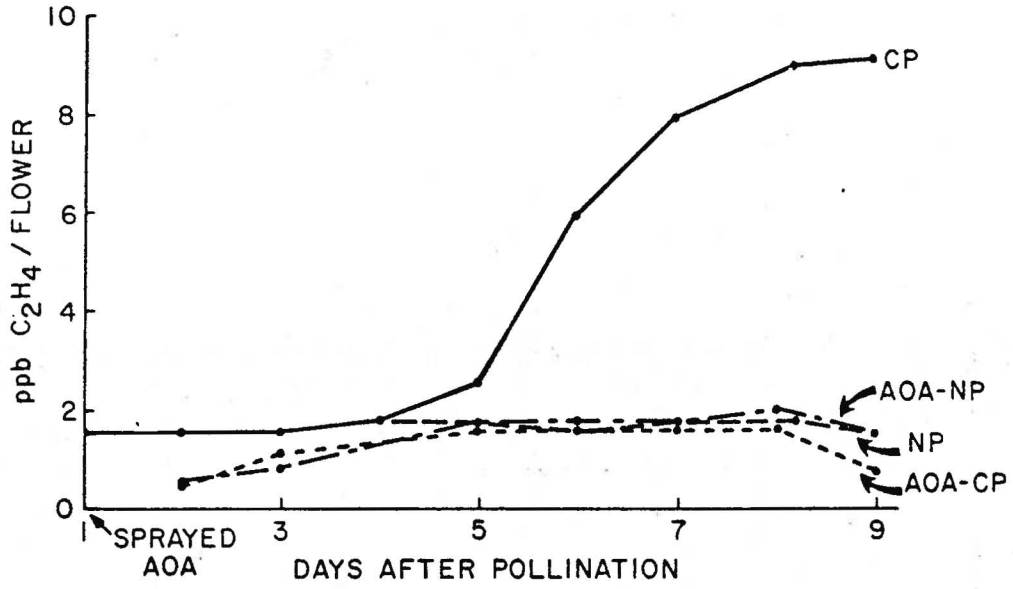

FIG. 2 Ethylene evolution by pollinated and nonpollinated almond flowers. Cross and nonpollinated flowers sprayed with 10 mM AOA, (aminooxyacetic acid, an ethylene synthesis inhibitor), produced amounts of ethylene similar to those seen in unsprayed, nonpollinated flowers. Embryo sac lengths in AOA-treated, cross-pollinated flowers were double those of AOA-treated, nonpollinated flowers (data not shown).

MEAN EMBRYO SAC LENGTHS OF FUNCTIONAL OVULES (um)

	NONPOLLINATED		SELF-POLLINATED		CROSS-POLLINATED	
	$\bar{x}$	sem*	$\bar{x}$	sem	$\bar{x}$	sem
Control	105.5	18.3	120	43.3	235	73.2
AOA	116.2	21.3	105.6	16.3	160	65.3
Eth	307.5	152.1	282.5	74.4	960	482.2
NAA	255	51.8			305.5	73.4
2-NAA	195.7	26.9				
CMPA	298	42.2				
TCA	245	131.8				
GA 100	295	89.7				
GA 250	310	47.6				
GA 500	546.7	168.4				
pierced	247.5	37.6				

*standard error of mean

FIG. 3. Embryo sacs of non-abortive ovules from treated flowers were measured in length, which is an indicator of degree of development. Means within groups indicate that all treatments, with the exception of AOA (aminooxyacetic acid), stimulated ovule development in non-pollinated flowers.

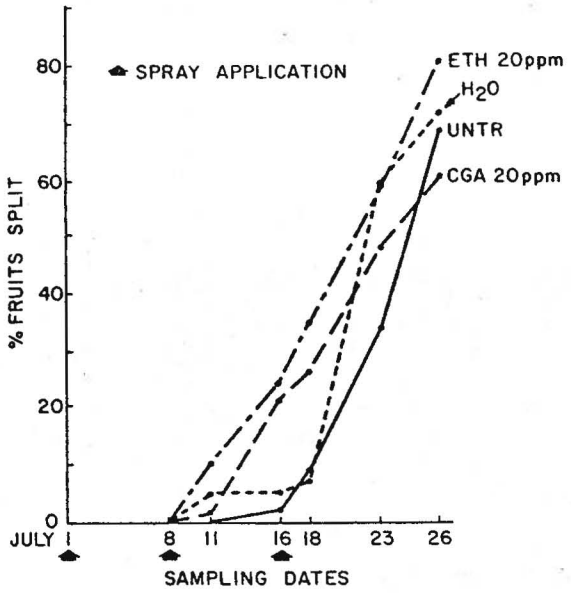

FIG. 4 In 1985, small-scale spray trials in Davis suggested a moderate effect of ethylene-releasing compounds on flower maturation.

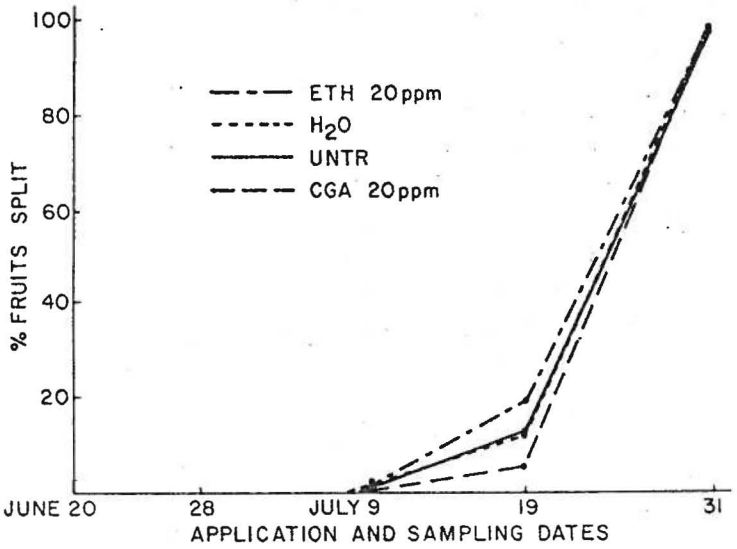

FIG. 5 1985 observations showed no effect on rate of maturation using 20 ppm CGA-15281 and 20 ppm ethephon on young Nonpareil trees in Durham when whole tree spray applications were made with a hand gun sprayer. (Co-operators: Joe Connell, Farm Advisor; Sam Lewis, Grower).

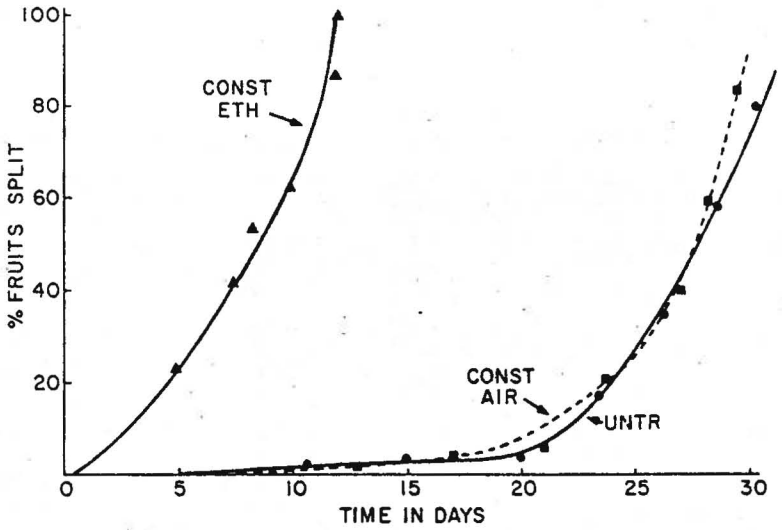

FIG. 6 Clusters of Nonpareil almonds were enclosed in bags which were flushed with air or air plus ethylene. Nuts were checked for dehiscence at regular intervals. Ethylene clearly promoted maturation.

DATE OF SPRAY	TREATMENT	% DROP
7/18	H_2O	88*
	100 ppm Eth	82*
	H_2O	92
	100 ppm Eth	93
7/25	H_2O	91
	200 ppm Eth	93
8/1	300 ppm Eth	88
	H_2O	83**
	200 ppm Eth	85**

* trees located on east end, outside row

** means of % drop of 6 sample trees

FIG. 7 In 1986, large scale spray trials in Woodland did not result in an increase in percent drop of ethephon-treated nuts over that of water-sprayed controls. (Co-operators: Wilbur Reil, Farm Advisor; Harry Dewey, Grower).

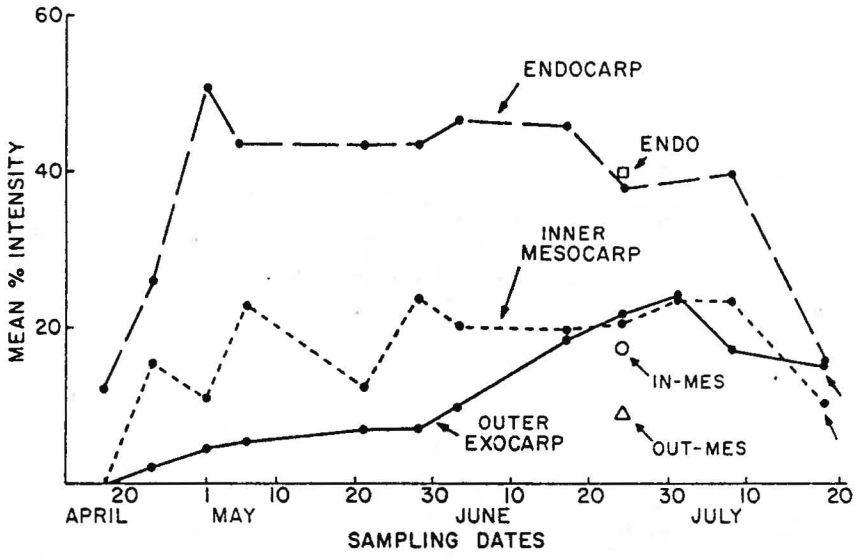

FIG. 8 Samples of almond hulls were analyzed throughout the maturation period (April-July, 1985). Biochemical tests for cell wall-degrading enzymes reported in gum duct formation and fruit ripening were made. The appearance and disappearance of pectins in the region of the dehiscence zone were measured as "Mean % Intensity" through microspectrophotometry. The sharp decline in this value in both exocarp and mesocarp regions coincides with a relatively large amount of pectinase measured in these tissues on July 18, 1985, (arrows). Levels of pectins were considerably lower in the exocarp and mesocarp of ethylene-gassed fruits sampled on June 24, 1985 (EXO, MESO), as compared to untreated samples collected on the same day. The level of pectin in the endo-carp (shell) tissue (ENO) was unaffected by the gassing.

	PAS	TBlueO	$HaFeCl_3$	RR	Cori
10 days post-anth	+	+	+	+	—
4/18/85	+	+	+	+	+
7/1/85	↓	↓	+	↓	↑
7/18/85	↓	↓	trace	↓	↓
ethylene gassed	↓ or —	↓	trace	trace	↓
crude pectinase	NA	—	NA	—	—

FIG. 9 The development of the dehiscence zone from ten days after anthesis to hullsplit was followed using cytochemical stains in conjunction with crude and purified degradative enzymes found to be associated with similar cellular changes in different developmental processes. Ethylene-gassed nuts were compared to untreated controls. The polysaccharide nature of the cell walls and "cementing" substances between cells appeared to be primarily pectinaceous, as evidenced by results seen here. The stains used were: PAS (periodic acid - Schiff's reagent), T Blue O (toluidine blue O), $HAFeCl_3$ (hydroxylamine-ferric chloride), RR (ruthenium red), and Cori (Coriphosphin).