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Canker Complex in Almonds
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Objectives: (1) To refine experimental methods to better detect the presence of Ceratocystis in cankers; (2) to evaluate chemical/biological agents for protecting fresh bark injuries from infection; (3) to evaluate chemical/biological agents for their efficacy in eradicating the fungus in diseased tissues; (4) to continue studies on the basis for wound resistance development; (5) to establish cause for apparent pruning wound infections; (6) to continue studies on the etiology of foamy canker disease.

Interpretive Summary: During 1983, studies were conducted which strongly suggested that the cause of pruning wound cankers in almond is the fungal pathogen, Phytophthora syringae. Isolations from cankers in trees in San Joaquin County and morphological and growth characteristics in culture were used to confirm the identity of the causal agent. Pathogenicity of an isolate was established by inoculating pruning wounds in Nonpareil and Ne Plus Ultra trees in February. By May, large gumming cankers were observed extending in all directions from the site of inoculation and Phytophthora syringae was reisolated from the margins of these cankers. Inoculation of pruning cuts with Ceratocystis fimbriata, the cause of mallet wound canker, indicated that this fungus was unable to significantly invade these wounds. We are developing a cut twig assay to better study these canker diseases under controlled conditions. So far, the results look promising and indicate that P. syringae is highly pathogenic at ambient temperatures between 2°- 20°C. Twigs from var. Mission appear to be less susceptible to this pathogen than twigs from Nonpareil and Ne Plus.

We have observed that bark wounds become resistant to infection by C. fimbriata 8-10 days after the injury. Microscopic examination of the tissues surrounding bark wounds revealed the formation during wound resistance development of a suberized and lignified periderm, a zone of tissue which may provide a barrier to invasion by Ceratocystis. Our goal is to try to exploit this phenomenon to achieve control of Ceratocytis canker.

Several experiments conducted during 1983 were either inconclusive or negative. For example, a field trial of Dichlorvos, an insecticide very active in the laboratory against vectors of Ceratocystis and Hull Rot organisms, did not significantly reduce populations of Drosophila flies. Populations of Nitidulid beetles in this experiment were too low to derive meaningful conclusions from the results.

Experiments during 1984 will examine the epidemiology of Phytophthora syringae and will focus on the susceptibility of pruning wounds and other tissues to this pathogen. We will continue our studies on wound resistance to Ceratocystis fimbriata.

Experimental Procedure:

I. Studies on the Aerial Phytophthora Canker

A. Association of Phytophthora syringae with apparent pruning wound cankers.

P. syringae was isolated from several cankers on limbs of trees in an orchard in San Joaquin County in May, 1983. The grower had numerous cankers in this orchard and most of these, but not all, appeared to be associated with pruning cuts. This association with pruning wounds is consistent with observations made in other orchards in the State (eg. Chico State University Varietal Trial, Butte County) and is a feature which distinguishes the aerial Phytophthora canker from Ceratocystis canker. The grower in this orchard had to prune out many limbs that had been girdled by the disease during spring. Most of these limbs were relatively young and usually did not exceed 7.6 cm (3 in.) in diameter. Nevertheless, the grower had lost production wood from this disease.

The pathogen was isolated from the canker margins by plating bark pieces on PV medium which is selective for Oomycetous fungi. Mycelium from the margins of colonies was transferred to amended lima bean agar (ALBA) and the fungus maintained at 18°-20°C on this medium. Identification was based on colony morphology on ALBA and corn meal agar, sporangia characteristics, growth in culture at various temperatures, and comparison of these characters with those of an isolate of P. syringae (F-78) from Dr. J. Mircetich. Inoculation of almond twigs with our isolate and subsequent reisolation from canker margins proved pathogenicity and satisfied the criteria for Koch's postulates. This isolate was used in subsequent experiments (henceforth designated as isolate F-79).

In February, 1983, an experiment was initiated to determine the relative abilities of P. syringae (Isolate F-79) and Ceratocystis fimbriata to infect pruning cuts. Pruning cuts with an exposed surface of approximately 2.54 cm (1 in.) in diameter were made on var. "Ne Plus" and "Nonpareil" trees. Agar plugs (3 mm in diameter) were obtained from the margins of culture, pressed onto the wound surface and then covered with tape. After 3 months, the trees were reexamined for canker development.

B. The effect of temperature on canker development with four Phytophthora species.

Four Phytophthora species (P. cactorum, P. citricola, P. megasperma, and P. syringae) isolated from cankers in almond trees were grown on an amended lima bean agar for use as inoculum. Almond branches (var. 'Drake') were cut, using pruning shears, into twigs approximately 15 cm. long (diameter 1-2 cm.). An agar plug (8 mm. in diameter) taken from a growing colony was placed on one cut end. Both ends were wrapped with parafilm. Three replications were done in coldrooms at four temperatures (2°C, 7°C, 12° C, 20°C). The canker length was measured weekly for 5 weeks.

C. Phytophthora canker development on three almond cultivars.

Branches from three almond cultivars ('Nonpareil', 'Mission', and 'Ne Plus Ultra') were cut, using pruning shears, into twigs approximately 15 cm. long (diameter 1-2 cm.). Phytophthora springae was grown on an amended lima bean agar for use as inoculum. An agar plug (8 mm. in diameter) from the fungal colony was placed on one cut end of the twig and then both ends were wrapped with parafilm. The experiment was performed twice. Each time 5 replications were placed in coldrooms at three temperatures (7°C, 12°C, 20° C). Twenty-one days after inoculation the advance of discoloration was measured.

II. Studies on Ceratocystis canker

A. Field, laboratory and microscopic studies on the development of resistance to C. fimbriata in almond bark wounds.

Previous observations indicated that wounded bark became resistant to infection within 14 days and, in some cases, as soon as 8 to 10 days after injury. We conducted several types of experiments during 1983 to better understand the basis for this phenomenon.

To assess wound resistance development under field conditions, branches (1.2-5.0 cm in diameter) were selected at random on var. Drake, Nonpareil and Long IXL trees at the Armstrong Experimental Farm, U.C. Davis. On each branch, 3-5 wounds were made with a #3 cork borer and the bark disk removed down to the cambium. In experiments with var. Drake and Nonpariel, wounds were made and then aged for 0, 2, 4, 6, 8, 10, 12, 14 or 20 days prior to inoculation with C. fimbriata. Wounds were inoculated by using a small paint brush to apply spores from a 10^6 conidia/ml suspension. The treated wound was then wrapped with parafilm. The developing cankers were examined 1 month after inoculation of the 20 da old wounds and the extent of necrosis measured after cutting away the bark. Although our protocol resulted in producing cankers that were of different ages at the time of evaluation, our primary objective here was to use the inoculated wounds for corroboration with microscopic observations of the development of resistance in uninoculated wounds. Wounds from var. Nonpareil were sectioned and examined microscopically during the aging period to ascertain the nature of the histological changes that had occurred at the time of inoculation. Samples were immediately sectioned using a freezing microtome, stained with histological dyes and examined. Representative sections were photographed with and without stains. Stains used were phloroglucinol/HCl for the detection of lignin or lignin-like material, Sudan Black B for detection of suberized tissues, and toluidine blue for general structural features.

In an effort to better study wound resistance development under controlled environmental conditions, we explored the use of 10-15 cm long, 1-2 cm diameter sections cut from branches. The ends of these were sealed with hot wax and sets were placed according to size in crispers containing a damp paper towel. Wounds were made at 2 day intervals over a 14-day period with a #2 cork borer, one wound per cutting. On the last day (0 days healing), each wound was inoculated with 10^5 conidia of C. fimbriata and then the cuttings were maintained in the crispers for two weeks at room temperature. Cankers were measured as above. In addition, the percentage of wounds that became infected was noted and the mean canker size for some treatments was determined both with and without the values for uninfected

wounds (0 cm) as indicated in the results. In some experiments, branches which had been stored in a coldroom at 35°C for one month or more were used as a source of cuttings. Sections were made of the wounds in the cuttings and examined histologically as above.

B. Attempt to reduce populations of insect vectors of Ceratocystis canker and Hull Rot organisms

A field trial of Dichlorvos, an insecticide very active in the laboratory against vectors of Ceratocystis and Hull Rot organisms, was undertaken in cooperation Dr. J. M. Ogawa who obtained an experimental use permit from the EPA. The test was made at the Tenneco Ranch near Fresno, CA. Two sprays were applied - one on July 27 (Hull split) and one on August 9. The experimental area consisted of 6 blocks, 3 received the insecticide and 3 were left unsprayed as controls. Each block was approximately one acre in size. The insecticide was applied by air blast sprayer, 2 lb ai./acre at a rate of 100 gal/acre. Traps (courtesy of Dr. E. Soderstrom, USDA, Fresno, CA) containing figs for sampling populations of Nitidulid beetles and Drosophila flies were distributed 15 to the block, and spaced on alternate trees in three rows. Insects were collected usually on a weekly basis and counted in the laboratory. In addition, insects were placed on carrot slices which are selective for C. fimbriata to determine if they were carrying spores of the pathogen.

III. Etiology of Foamy Canker

An experiment was conducted to determine if the combination of high temperature stress and inoculation with the Zymomonas sp., the organism frequently isolated by Dr. Beth Teviotdale and us from the sap of Foamy canker diseased trees, would reproduce disease symptoms. Inoculation of nonstressed trees with this organism have so far been unsuccessful.

One year old trees (var. Carmel on Nemaguard rootstock) in pots were subjected to a 38°C day (10 hr) - 27°C night (14 hr) temperature regime in a growth chamber for various periods of time. Trees were inoculated through bark flaps along the main stem by placing bacteria scraped from colonies on agar with a cotton swab on the exposed cambial surface. In addition, droplets of a thick bacterial suspension were placed on leaf axils and tips and internodes and then stab inoculated. The isolate used for inoculation was grown on YSC (yeast-sucrose-carbonate) slants at room temperature for 3 days. Controls were treated similarly except water was used in place of the bacteria. Eight trees were used per treatment - five received inoculations and three served as controls. After inoculation, trees were placed in a lathhouse or stressed in the growth chamber for various periods of time until removal to the lathhouse. Trees were observed 3 weeks after the start of the experiment and periodically for several weeks thereafter.

The treatment schedule was as follows:

Treatment No.							
1	Day 0/Inoculate	Lath House					Day 21
2	"	Stress	Day 3	Lath House			"
3	"	Stress	Day 6	Lath House			"
4	"	Stress	Day 9	Lath House			"
5	"	Stress	Day 14	Lath House			"
6	Stress	Day 7/Inoculate	Stress	Hi Stress			"

To determine if a preinoculation stress was necessary for symptom development, trees in treatment 6 were placed in the growth chamber for seven days prior to inoculation. They were then immediately returned to the chamber. During the last seven days in the chamber, these trees were exposed to a high-stress regime of 40°C day and 27°C night and then placed in the lathhouse.

Results

I. Studies on the Aerial *Phytophthora* Canker

A. Association of *Phytophthora syringae* with apparent pruning wound infections.

Only the pruning cuts inoculated with *P. syringae* developed extensive, gumming cankers. *Phytophthora syringae* was reisolated from all of these cankers. Pruning cuts inoculated with *C. fimbriata* did not develop cankers and necrosis was observed only a few millimeters into the cut.

B. The effect of temperature on canker development with four *Phytophthora* species.

The mean canker expansion rates (canker length divided by days after inoculation) are given in Table 1. The cankers produced by *P. syringae* expanded significantly and substantially faster at 2°C and 7°C than those produced by the other *Phytophthora* species tested. At 2°C, only *P. syringae* produced measurable cankers. At 12°C the difference between the size of cankers produced by *P. syringae*, *P. cactorum*, and *P. citricola* was slight and not statistically significant. Although there is substantial difference in the mean canker expansion rates for cankers produced by the various *Phytophthora* species at 20°C, the differences are not statistically significant because of unusually large variability.

C. Phytophthora canker development on three almond varieties.

The results are given in table 2. At all three temperatures tested, the cankers expanded significantly slower on Mission than on 'Nonpareil' or 'Ne Plus Ultra', whereas there was no significant differences ($P = 0.05$) between 'Nonpareil' and 'Ne Plus Ultra'.

II. Studies on Ceratocystis canker

A. Field, laboratory and microscopic studies on the development of resistance to C. fimbriata in almond bark wounds.

Microscopic examination of transverse sections of healing wounds revealed definite morphological and histochemical changes occurring, although the time at which these changes occurred was somewhat variable among samples within a two to four day period. Within four to six days after wounding, there appeared the first signs of a differentiated zone of cells transversing a diagonal from the wound edge at the vascular cambium to an area in the phelloderm within a few millimeters of the wound edge. The cells in this zone after 8-10 days of wound-healing had phloroglucinol/HCl positive material deposited on the side facing the wound surface. The cell walls of this zone were stained by Sudan Black B, indicating the deposition of suberin or suberin-like material. By 12 to 14 days after wounding, this cell layer had become a clearly defined wound periderm with suberized cell walls and lignin/lignin-like material deposited on the outside of this new layer and towards the wound surface. Color photographs of this will be submitted separately.

The time for the occurrence of these events coincided with the development of resistance observed in the field by Moller and DeVay during the 1960's and by us in 1982. However, the wounds made in the parallel experiments this season on Nonpareil and Drake branches did not develop resistance as observed previously. This may have been due to our method of inoculation whereby the wounds were covered with parafilm after applying a rather concentrated spore suspension and this may have overwhelmed the resistance.

Experiments in which we used the branch sections were somewhat variable. In one experiment with cuttings from var. Drake, the pattern of resistance development was consistent with our earlier field observations and fewer cankers developed from wounds that had healed for 10 to 14 days prior to inoculation (Table 3). This reduction during wound healing in the percentage of successful infections was observed in branch sections of Nonpareil, Ne Plus and Mission. Preliminary microscopic observations of the healing wounds from these branch sections revealed the formation of suberized and lignified cell layers similar to that in branches on intact trees.

To see if time of storage of the branches affected the results in the laboratory crisper assay, sections from branches that had been stored at 35°C for 3 days or 6 weeks were inoculated. All of the 3 day-stored branches became infected whereas only 66% of the wounds in branches stored for 6 weeks became diseased. In addition, canker size in the branches

stored for 6 weeks that became infected was significantly smaller than in the ones stored for 3 days (1.1 ± 0.1 cm and 1.6 ± 0.1 cm, respectively).

B. Attempt to reduce populations of insect vectors of Ceratocystis canker and Hull Rot organisms.

Dichlorvos did not appear to have any effect on populations of Drosophila flies (Fig. 1) or Nitidulid beetles (data not shown). The population of Nitidulid beetles was extremely low in this orchard and all isolations for C. fimbriata were negative.

III. Etiology of Foamy Canker

None of the trees inoculated and exposed to the high temperature became diseased.

Discussion

Phytophthora syringae seems well adapted in growth and development in almond tissues under the common winter conditions of the central valley of California of low temperatures and high rainfall. At temperatures as low as 2°C, P. syringae could still cause cankers, whereas the other Phytophthora species pathogenic on almond could not. These observations are consistent with growth of these fungi on agar media wherein P. syringae can grow better at low temperatures, but not as well at high temperatures relative to these other Phytophthora species.

The use of branch sections in plastic crispers seems to work well for the Phytophthora canker studies and the fungus moves rapidly in these tissues. Mission shows some resistance in this assay (smaller cankers or no cankers at all) relative to Nonpareil and Ne Plus. Although the assay is somewhat unnatural, field observations at the Chico State University Variety trial do support this contention. Experiments are underway to more definitively establish the relationship of results with branch cuttings to those obtained with intact trees. We are currently conducting studies on varietal differences in susceptibility in the field, as well.

Field observations and the experiments discussed strongly suggest that a primary avenue for infection by P. syringae is through pruning wounds. We are very interested in this aspect particularly with respect to inoculum levels and time of wetting necessary for infection of pruning wounds, and the time that pruning wounds remain susceptible during the fall, winter and spring. Such information could conceivably be integrated into a dormant spray program designed for control of other almond diseases but which may also provide protection of pruning cuts of highly susceptible varieties from P. syringae. If pruning wounds become resistant to infection with time, then it would also be of interest to know if the basis for this resistance is similar to that which occurs in bark wounds against Ceratocystis. Certainly these pathogens are quite different in their mode of infection as well as in other aspects of their biology but the kinds of changes under investigation (i.e. formation of a lignified and suberized wound periderm) probably contribute to host resistance to both organisms.

The suberin and lignin-like material observed in healing bark wounds constitute polymers which are very difficult for fungal pathogens to degrade and penetrate. The studies on this in relation to Ceratocystis canker are encouraging and provide us with clues as to the basis for wound resistance. Nevertheless, further work is necessary to address some of the inconsistencies in our data from 1983. The use of branch sections may be of utility in Ceratocystis canker studies provided some of the variables such as storage life and bark moisture content can be identified and controlled.

I am unaware of any chemical treatment that is effective in eradicating a fungal pathogen from cankers in thick barked trees such as Prunus spp. There are some experimental biological control agents that have been used but these are highly specific to certain host-pathogen interactions. It may be that surgery or modified surgery of existing cankers will be our only recourse for eradication but the information on wound-healing dynamics and rate of canker expansion will impact directly on the successful implementation of such an approach.

Control of the insect vectors of C. fimbriata seems difficult, if not impossible. The results with Dichlorvos were discouraging and difficult to draw definitive conclusions. The populations of Nitidulids were surprisingly low, although in some years populations may not reach high levels until October (Dr. E. Soderstrom, personal communication). Since we stopped sampling in mid-September, this might explain the results. It still may be worthwhile to examine the efficacy of fungicidal protection of bark wounds at the time of injury. Again, our data on wound resistance dynamics will directly impact on such investigations because they indicate how long a treatment must be effective before the tree can seal off the injury. Our plants for 1984 are to investigate this avenue of research.

The Tenneco Ranch orchard contained many trees with cankers. Isolations from these for Ceratocystis fimbriata were all negative but they appeared similar to that for typical Ceratocystis cankers. Many cankers appeared to be a result of aerial Phytophthora infections, also, but isolations were negative for this organism as well. (Not surprising because P. syringae will die during the hot summer months.) We have repeatedly experienced this in other orchards although our success in isolating from Ceratocystis cankers initiated by us is close to 100%. What this attests to is the difficulty in confidently distinguishing these two diseases. Although no systematic survey has been performed, our collective observations during the past two years suggest that the aerial Phytophthora canker is the more common of the two diseases. We are not discounting, however, the possibility that our sampling techniques may need modification or that the behavior of C. fimbriata in almond is now different in some way from what it was during the 1960's.

The negative data from the Foamy canker experiment merely indicate that the disease is more complex than the interaction of just the two variables tested.

Experiments during 1984 will examine the epidemiology of P. syringae and will focus on the susceptibility of pruning wounds and other tissues to this pathogen. We will continue our studies on wound resistance to C.

fimbriata as outlined above. No further work is planned for the Foamy canker disease because I believe this would diminish our efforts on the aerial *Phytophthora* and *Ceratocystis* canker problems.

Table 1. Canker development at four temperatures in detached almond twigs (cv. 'Drake') infected with various Phytophthora species pathogenic on almond.

	Canker expansion rate (mm/day)			
	Temperature (C°)			
Species	2°	7°	12°	20°
P. syringae	1.1 a[1]	1.3 a	2.9 a	3.1 [2]
P. cactorum	0.0 b	0.0 c	3.6 a	4.8
P. citricola	0.0 b	0.5 b	3.3 a	6.8
P. megasperma	0.0 b	0.4 b	0.3 b	0.8

[1] Numbers followed by the same letter are not significantly different (P = .05) according to Duncan's multiple range test.

[2] No significant differences (P = 0.05).

Table 2. Phytophthora canker development in three almond varieties[1]

Variety	Canker expansion rate (mm/day) Temperature (C) 7°	12°	20°
Ne Plus Ultra	1.3 a[2]	2.8 a	2.0 a
Nonpareil	1.0 a	2.7 a	3.0 a
Mission	0.6 b	1.2 b	0.8 b

[1] Detached twigs were inoculated with Phytophthora syringae and then incubated at the indicated temperature.

[2] Numbers followed by the same letter are not significantly different (P = .05) according to Duncan's multiple range test.

Table 3. Ceratocystis canker development in healing wounds on var. Drake branch sections.

Age of wound at time of inoculation (da)	% Wounds infected	Length (cm)[1]	Length (cm)[2]
0	100	2.8 ± 0.6 A	2.8 ± 0.6 A
2	100	2.0 ± 0.3 AB	2.0 ± 0.3 AB
4	100	2.5 ± 0.3 A	2.5 ± 0.3 A
6	100	2.2 ± 0.1 AB	2.2 ± 0.1 AB
8	100	1.3 ± 0.2 BC	1.3 ± 0.2 B
10	40	0.9 ± 0.2 C	1.4 ± 0.1 B
12	40	0.8 ± 0.2 C	1.2 ± 0.3 B
14	40	0.9 ± 0.2 C	1.4 ± 0.2 B

[1] Values listed are the mean - S.D. for canker lengths measured two weeks after inoculation. Calculation of the values in this column included 0 values for wounds which were completely resistant.

[2] Values in this column did not include 0 values in the calculations.

[3] Values followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

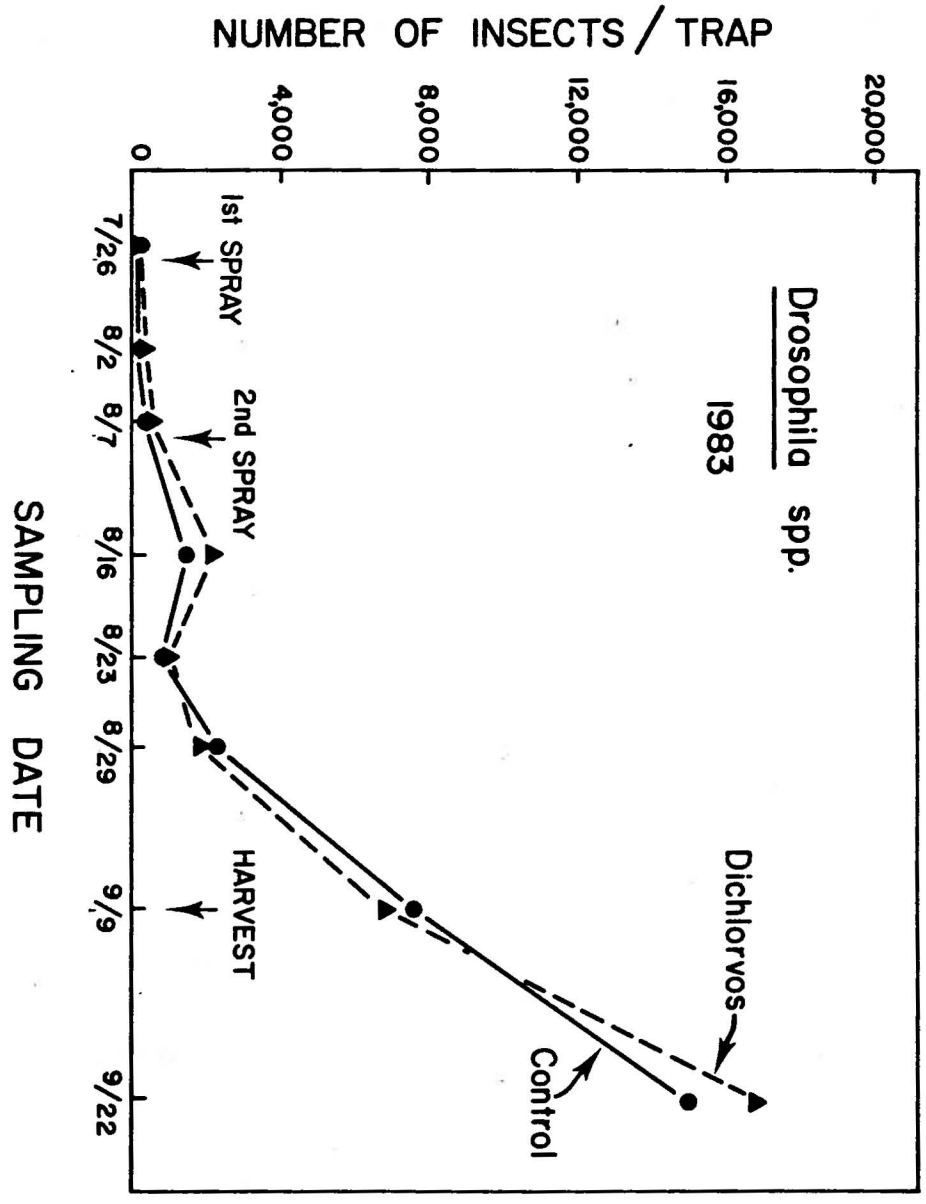

Fig. 1. Response of Drosophila fly population to Dichlorvos at the Tenneco Ranch Orchard, Fresno, CA.