Annual Report 1996

Prepared for the Almond Board of California

Project No. 95-JA1: **Management of Almond Anthracnose in California**: Detection and Identification of the Causal Pathogen, Epidemiology, and New Management Practices.

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SUMMARY

In 1996, almond anthracnose caused by *Colletotrichum acutatum* was widespread in the northern Sacramento and central San Joaquin Valleys of California. Susceptible almond cultivars included: Butte, Carmel, Fritz, Harvey, Mission, Monterey, Peerless, Price, Thompson, NePlus Ultra, and Nonpareil. Nonpareil was the least susceptible of the cultivars evaluated. Symptoms of the disease included sunken orange lesions of fruitlets. Infections during latter stages of fruit development often produced profuse gumming. Leaves of infected spurs yellowed, wilted, and died. Often, dieback of spurs and branches occurred. Thus, the disease caused direct losses of crop by infecting almond fruit and indirect losses by causing shoot and branch dieback of trees. In our studies, the fungal pathogen has been isolated from diseased blossoms, fruit, peduncles, spurs, and from overwintering mummies. Isolations from diseased leaves and wood, however, were less successful although extensive branch dieback and leaf yellowing occurred. The fungal pathogen was additionally characterized using morphology, molecular techniques, and fungicide sensitivity assays. Using random and single repeat primers, two distinct sub-populations could be identified among the almond isolates of *C. acutatum*. Additionally, there were differences between the two populations in pigmentation of recently isolated cultures (pink vs. gray) and sensitivity to benomyl at low conidial concentrations ($< 3 \times 10^5$ conidia/ml) using filter-paper disk assays. Extensive laboratory and field evaluations for the development of disease management strategies were conducted. Fungicides evaluated included: azoxystrobin, fenbuconazole, myclobutanil, propiconazole, tebuconazole, iprodione, captan and thiophanate-methyl. Propiconazole and tebuconazole were the most effective compounds in field evaluations. Currently, propiconazole (Section-18 granted for 1997) and captan are being used to manage almond anthracnose in California.

INTRODUCTION

In the spring and summer of 1995 and 1996, a serious epidemic of almond anthracnose occurred in the Sacramento and San Joaquin Valleys. The disease is caused by the fungus *Colletotrichum acutatum.* Symptoms included blossom blight, fruit infections with initial symptoms appearing as orangish, circular, and sunken lesions that often gummed in advance stages of infection (Fig. 00), and branch dieback with associated leaf yellowing, wilting, and death. Fruit often did not drop and the infections continued to develop into the spurs and shoots. The disease was similar to brown rot in causing spur infections that originated from blossom or fruit infections. Fruit symptoms were observed 2-3 wk after petal fall. Fruit eventually shriveled up but remained light rusty orange and appeared like unfertilized almond fruit (blanks). Throughout the spring and summer, as conducive environments continued, infections developed on mature fruit and branch dieback became apparent as temperatures increased and the fungus advanced into woody tissues.

Our research objectives included the characterization of the anthracnose pathogen by growth studies and molecular characterization. Disease management strategies critically depend on proper pathogen identification. In the past few years molecular methods have become available that can assist in the identification of species and establish genetic relationships to other species. In addition, genotypes within a species that exhibit limited DNA sequence variations can be differentiated. Many of these new methods involve the polymerase chain reaction (PCR). With this technique, DNA can be analyzed with high sensitivity using crude extracts from small amounts of sample material. Using this technology, our aim was to characterize the anthracnose pathogen, verify its species identity and study its population structure.

Additionally, we are developing management strategies for almond anthracnose in California. Management programs that minimize the potential development of resistant populations are being evaluated. These programs include rotation of single and multi-site mode of action fungicides and cultural practices that minimize conducive environments and sources of inoculum. In 1996, our immediate goal was to develop identify efficacious fungicides against the disease using laboratory and field studies and to help in the emergency registration of an effective fungicide.

OBJECTIVES

I. Collection of isolates
   - A. Disease survey in major almond growing regions within California
   - B. Isolation and culturing of causal anthracnose pathogen from almond tissues

II. Identification of *Colletotrichum* spp.
   - A. Cultural studies on morphology
   - B. Molecular characterization
   - C. Detection of *Colletotrichum* species in plant tissue using molecular probes

III. Preliminary management strategies
   - A. *In vitro* sensitivity of fungal isolates to selected fungicides
   - B. Development of control strategies

MATERIALS AND METHODS

Fungal isolation, culturing, and morphology. Infected tissue was surface-sterilized with 400 ppm bleach for 1 min and plated onto potato dextrose agar (PDA) amended with rifampicin and ampicillin to inhibit bacterial growth. Cultures were maintained on PDA. Isolates were characterized using conidial morphology, temperature relationships (10, 15, 20, 25, 30, and 35 C for 5 days), molecular techniques, and fungicide sensitivity. Conidial morphology of isolates grown on PDA, pea straw agar, and on almond fruit was variable in size and shape.

Evaluation of* in vitro *fungicide sensitivity. For the filter disk assay, the surface of PDA was inoculated with spore suspensions. Then fungicide-treated filter paper disks were placed on top. Inhibition zones around the filter paper disks were recorded after three days. For radial growth inhibition studies, petri dishes with fungicide-amended media were inoculated with mycelial agar plugs and fungal growth was measured after 6-8 days. The effect of fungicides on spore germination was evaluated by placing drops of spore suspensions onto the surface of fungicide-amended agar. Spore germination was recorded after 16-20 hr and colony establishment was observed after 1-4 days. Fungicides evaluated included: azoxystrobin (Abound 80WG), fenbuconazole (Indar 75WP), myclobutanil (Rally 40WP), propiconazole (Orbit 45WP), tebuconazole (Elite 45DF), captan (Captan 50WP), chlorothalonil (Bravo), benomyl (Benlate 50WDG), and thiophanate-methyl (Topsin 75WP). Rates ranged from 0.1 to 1200 µg/ml depending the assay used.

Molecular characterization of* Colletotrichum *species. *Colletotrichum* DNA was isolated using a modification of the Raeder & Broda (1985) method. For species identification, species-specific primers for *C. gloeosporioides*, *C. acutatum*, and *C. fragariae* (Mills *et al.*, 1994) were used in DNA amplification reactions. To detect DNA variation between isolates, two DNA repeat sequences $((cag)_5$ and $(gaca)_4$, Freeman *et al.*, 1996) and three random 10mer oligonucleotides were used as primers. Following PCR amplification, DNA products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator.

Evaluation of fungicides for management of almond anthracnose in the field. Two plots were established in commercial orchards: one in Butte Co. and the other in Merced Co. Disease management strategies were conducted with azoxystrobin (Abound 80WG - 2.4 oz/A), fenbuconazole (Indar 75WP - 2 oz/A), myclobutanil (Rally 40WP - 6 oz/A), propiconazole (Orbit 40WP or 3.6EC - 4 oz/A), tebuconazole (Elite 45DF - 8 oz/A), iprodione (Rovral 50WP - 1 lb/A), captan (Captan 50WP - 9 lb/A) and thiophanate-methyl (Topsin 75WP - 2 lb/A). Treatments were applied using an air-blast sprayer calibrated for 100 gal/A. Timing was based on phenological stage: pink bud, full bloom, and 1- & 2-wk after petal fall. In each plot there were four single-tree replications for each treatment. Treatments were completely randomized in a single tree row with buffer rows on either side. Evaluations were made in mid-March and in mid-April. For this, 200 fruit (on individual spurs) per replication were evaluated and disease was expressed as the incidence of anthracnose per total fruit evaluated.

RESULTS AND DISCUSSION

I. Disease symptoms and collection of isolates.

Symptoms included blossom blight, fruit infections, and spur and branch dieback. Initially, fruit symptoms appeared as orangish, circular, and sunken lesions (Fig. 00A-C) that often gummed in later stages of fruit development. Fruit infections began in the hull and progressed into the developing endosperm (Fig. 00C). Fruit often did not drop and the infections continued to develop into the spurs and shoots. Leaves on infected spurs often yellowed, wilted, and died from fruit infections that developed into spurs (Fig. 00A-B). Branch dieback with associated leaf yellowing, wilting, and death occurred latter in the season. The disease was similar to brown rot in causing spur infections that originated from blossom or fruit infections. Fruit symptoms were observed 2-3 wk after petal fall. Fruit eventually shriveled up but remained light rusty orange and appeared like unfertilized almond fruit (blanks).

In a disease survey that was carried out in the major almond growing regions within California, anthracnose was directly observed causing economic losses in five primary counties: Butte, Glenn, Merced, San Joaquin, and Stanislaus. Other counties where the disease was observed (personal communications) include Colusa and Sutter-Yuba (J. Edstrom), Fresno (B. Teviotdale), and Tehama (R. Buchner). Additionally, the disease was observed in Kern Co. but it was not causing crop losses. Almond isolates were collected from 30 orchards in the five primary counties from March to September 1996. Isolates have been obtained from blossoms, fruit, peduncles, spurs, leaves and wood of selected cultivars. Generally, isolations from leaf and wood tissue were much less successful in spite of extensive branch dieback and leaf yellowing. Isolations from infected immature fruit showing sunken, orangish lesions were highly successful. The almond cultivars include: Butte, Carmel, Fritz, Harvey, Mission, Monterey, Peerless, Price, Thompson, NePlus Ultra, and Nonpareil. Nonpareil was the least susceptibe of the cultivars evaluated. Other cultivars where the disease was observed include Merced and Padre. In February of 1996 isolations were carried out from fruit mummies collected in four orchards. *Colletotrichum* could be recovered from hull, shell and endosperm tissue. The recovery rate in two orchards (based on the number of mummies investigated) was 100%. In total, 260 *Colletotrichum* isolates from the tissues listed above were collected and characterized from 30 different orchards.

II. Identification of Colletotrichum *species.*

Cultural studies on morphology. Isolates were characterized using conidial morphology, temperature relationships, molecular techniques, and fungicide sensitivity. Conidial morphology of isolates grown on PDA, pea straw agar, and on almond fruit was variable in size and shape. Conidia in mass were orange or salmon colored, whereas, individual conidia were hyaline, aseptate, and fusiform measuring 8-17 x 2.5-4 µm. Generally, the conidial morphology of almond isolates was more similar to stock cultures of *C. acutatum* from strawberry and other stone fruit crops than to stock cultures of *C. gloeosporioides* from citrus and papaya that were grown on the same media. However, in comparisons between *Colletotrichum* species grown on pea straw agar, conidial size was overlapping. Therefore, we conclude that size and shape of conidia are not reliable criteria for identifying species of *Colletotrichum*. Based on temperature relationships of isolates grown at 10,

15, 20, 25, 30, and 35 C for 5 days, *C. gloeosporioides* had an optimum growth rate at 30 C and grew faster than *C. acutatum* isolates at their optimum of 25 C.

Molecular characterization and detection of* Colletotrichum *in plant tissue. Using species-specific DNA primers for *C. gloeosporioides*, *C. acutatum*, or *C. fragariae* in PCR amplification experiments, DNA from the almond isolates only yielded amplification products of the expected size with the *C. acutatum* primer. In addition, restriction fragment patterns of ribosomal DNA from the almond isolates were more similar to those of a reference culture of *C. acutatum* from strawberry than to those of *C. gloeosporioides*. These results indicate that the fungus collected from almond is *C. acutatum* and not *C. gloeosporioides*. In order to investigate the genetic diversity among the almond isolates, two DNA repeat sequences and three randomly selected 10mer primers were used in DNA amplifications. Compared to the species-specific primers, these latter primers are much more sensitive in detecting DNA differences. The results of these experiments demonstrated that two distinct sub-populations of *C. acutatum* exist on almond with one group forming the majority (60%) of the isolates. As an example, in Fig. 0 the electrophoretic separation of amplification products using one of the primers is shown. The two genotypes are very distinct and can be easily differentiated. The two sub-populations sometimes occurred in the same orchard and there were no distinct cultivar or geographical relationships. Using the species-specific primers, we were also able to directly verify the presence of *C. acutatum* in infected almond fruit by PCR amplification of total DNA using the species-specific primer. Thus, **rapid identification is possible from fruit samples without isolation of the fungus on cultural media**.

III. Preliminary management strategies

In vitro sensitivity of fungal isolates to selected fungicides. Chemical control strategies are dependent on the species of *Colletotrichum* (Bernstein and Miller, 1995). Thus, we evaluated almond isolates for their sensitivity to fungicides using three methods: filter-disk assay (which evaluates the effect on spore germination and colony formation), mycelial growth on fungicide-amended media, and spore germination on fungicide-amended media. Isolates of *C. acutatum* were less sensitive to benomyl than isolates of *C. gloeosporioides*, whereas isolates of both species were sensitive to captan (Fig. 1, Table 1). The fungicides chlorothalonil, benomyl, propiconazole, tebuconazole, fenbuconazole, myclobutanil, captan, and azoxystrobin were also evaluated. Using the filter-disk assay, **propiconazole, tebuconazole, and captan were the most effective against *C. acutatum*** (Tables 1-3), whereas propiconazole, tebuconazole, and benomyl were the most effective against *C. gloeosporioides* (Tables 1-3). The two sub-populations of *C. acutatum* described above differed in their sensitivity to benomyl. The difference, however, was dependent on spore concentration: isolates of both populations were completely insensitive to benomyl at spore concentrations greater than 3×10^5. In the fungicide-amended media studies, mycelial growth was inhibited similar to the disk assay. The results for benomyl, captan, myclobutanil, propiconazole, and tebuconazole are displayed in Table 4. It is evident that propiconazole and tebuconazole had very similar inhibitory effects against the two *C. acutatum* genotypes from almond when compared with *C. gloeosporioides*. However, captan and benomyl were more effective against *C. gloeosporioides* than to *C. acutatum* from almond. Conidial germination was not inhibited at concentrations less than 50 µg/ml of myclobutanil, propiconazole, tebuconazole, and benomyl for the almond isolates. Captan completely inhibited

germination at the same concentration. EC_{50} values are currently being developed for selected fungicides.

Development of management strategies. Standard fungicide trials to determine efficacy of selected compounds and correct timing of application were initiated in the spring of 1996. Two plots were established: one in Merced Co. (Fig. 2) and the other in Butte Co. (Fig. 3). Trials were conducted using an air-blast sprayer calibrated for 100 gal/A. Tebuconazole, propiconazole, fenbuconazole, myclobutanil, myclobutanil-captan, myclobutanil-maneb, azoxystrobin-captan, azoxystrobin-thiophanate-methyl, and iprodione-captan were evaluated as bloom and petal fall treatments. All treatments significantly reduced the disease, however, tebuconazole and propiconazole had the lowest incidence (Figs. 2-3). None of the treatments eradicated the disease and all performed as protectants. Once treatments stopped and conducive conditions continued, the disease subsequently increased (Figs. 2-3). Thus, fungicide programs are dependent on continued applications prior to conducive wet conditions throughout the spring. Currently, a Section-18 emergency registration for propiconazole has been approved by EPA. Rotation programs between fungicides of different classes (e.g., propiconazole, captan, etc.) will be evaluated in the spring of 1997 to devise management strategies to reduce the potential development of resistant populations of the pathogen.

Growers who suspect they have anthracnose in their orchards should contact their county farm advisor for identification of the disease. Investigations are currently being conducted to evaluate the effect of removal of mummified fruit and dead branches to reduce inoculum of the fungus.

REFERENCES

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Table 1. Mean inhibition zones of *Colletotrichum acutatum* and *C. gloeosporioides* identified by their original host to benomyl and captan.

Taxonomic Name	Original Host	Isolates*	Inhibition Zone Radius (mm)**			
			Captan		Benomyl	
C. acutatum	Strawberry	EEB 99	3.0 ± 2.19	a	0.04 ± 0.25	b
	Almond	JEA 92-8 JEA 92-18 JEA 95-B	4.05 ± 2.68	a	2.20 ± 3.95	b
	Peach	BM 95-1	4.02 ± 3.03	a	3.44 ± 3.36	b
C. gloeosporioides	Citrus	JMO-22	4.03 ± 2.67	a	14.0 ± 6.80	a
	Papaya	RJH-P4 RJH-P7	4.48 ± 3.73	a	11.27 ± 6.80	a

* - Potato dextrose agar plates were simultaneously inoculated with each isolate using 100 ul of a conidial suspension (30,000 conidia/ml), filter disks saturated in 0, 300, 600, or 1200 *u*g/ml were placed on the agar surface, and the plates were incubated for 3 days at 25ºC .

** - Inhibition zones are the average of all concentrations evaluated for each fungicide. Values followed by the same letter were not significantly different using Tukey's studentized range test ($P > 0.05$).

Table 2. Mean inhibition zones of *Colletotrichum acutatum* and *C. gloeosporioides* to selected contact and demethylation inhibitor fungicides.

Fungicide			Inhibition Zone Radius (mm)	
Trade Name	Chemical	Formulation	*C. acutatum*	*C. gloeosporioides*
Elite	Tebuconazole	45DF	12.5 a	16.8 a
Orbit	Propiconazole	3.6EC	10.3 b	12.4 b
Rally	Myclobutanil	40WP	4.1 c	6.8 c
Bravo	Chlorothalonil	720F	3.8 c	2.3 d
Abound	Azoxystrobin	75WP	0.75 d	0.8 de
Indar	Fenbuconazole	80WG	0.1 d	0.8 de
Check	---	---	0 d	0.0 e

* - Potato dextrose agar plates were simultaneously inoculated with each isolate using 100 ul of a conidial suspension (30,000 conidia/ml), filter disks saturated in 0, 300, 600, or 1200 ug/ml were placed on the agar surface, and the plates were incubated for 3 days at 25ºC .

** - Inhibition zones are the average of all concentrations evaluated for each fungicide. Values followed by the same letter were not significantly different using Duncan's multiple range test ($P > 0.05$). Inhibition zones for myclobutanil and benomyl were diffuse numerous colonies within a small zone.

Table 3. Mean inhibition zones of *Colletotrichum acutatum* to captan, benomyl, myclobutanil, and tebuconazole fungicides.*

Fungicide			Inhibition Zone Radius (mm)*** for selected almond isolates of *C. acutatum*		
Trade Name	Chemical	Formulation	23-1	24-1	92-6
Elite	Tebuconazole	45DF	19.4 a	13.8 a	12.2 a
Captan	Captan	75DG	8.6 b	8.3 b	8.3 b
Rally	Myclobutanil	40WP	3.1 c	3.1 c	3.1 c
Benlate	Benomyl	50WG	1.4 d	0.4 d	0.4 d
Check	---	---	0.0 d	0.0 d	0.0 d

* - Potato dextrose agar plates were simultaneously inoculated with each isolate using 100 ul of a conidial suspension (30,000 conidia/ml), filter disks saturated in 0, 300, 600, or 1200 ug/ml were placed on the agar surface, and the plates were incubated for 3 days at 25ºC .

** - Inhibition zones are the average of all concentrations evaluated for each fungicide. Values followed by the same letter were not significantly different using Duncan's multiple range test ($P > 0.05$). Inhibition zones for myclobutanil and benomyl were diffuse numerous colonies within a small zone.

Table 4. Relative inhibition of radial growth of *Colletotrichum acutatum* and *C. gloeosporioides* on fungicide-amended media

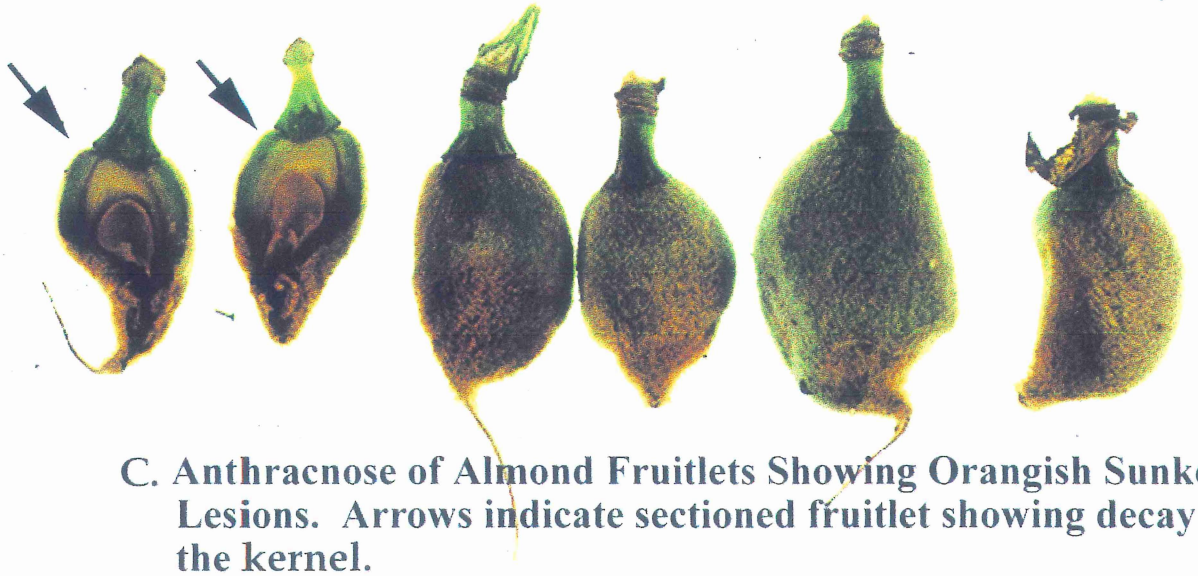
Fungicide	Conc. (*u*g/ml)	Percent Inhibition of Growth		
		C. acutatum from almond		
		genotype 1	genotype 2	*C. gloeosporioides*
benomyl	0.1	44.8 c	17.7 d	66.1 b
	0.5	57.9 ab	67.7 c	96.9 a
	1	56.7 b	72.4 b	97.7 a
	5	55.8 b	72.6 b	99.2 a
	10	54.8 b	72.3 b	100 a
	50	61.5 a	76.1 a	100 a
captan	50	30.1 c	26.1 c	68.4 b
	100	40.8 b	39.5 b	86.5 a
	500	70.0 a	77.2 a	97.0 a
	1000	74.1 a	84.8 a	93.7 a
myclobutanil	0.5	43.2 c	20.4 e	14.5 e
	1	52.7 c	31.7 d	29.8 d
	5	75.4 b	64.6 c	58.6 c
	10	79.5 ab	81.0 b	70.2 b
	50	95.8 a	95.1 a	92.4 a
propiconazole	0.1	25.3 e	24.9 e	23.9 e
	0.5	42.0 d	49.9 d	58.9 d
	1	56.4 c	58.2 c	68.6 c
	5	80.2 b	85.4 b	89.5 b
	10	79.5 b	92.4 a	97.0 a
	50	94.0 a	95.7 a	98.5 a
tebuconazole	0.1	33.8 e	32.2 e	20.6 e
	0.5	66.1 d	67.8 d	56.3 d
	1	75.0 c	80.0 c	64.7 c
	5	87.2 b	89.5 b	81.3 b
	10	95.8 a	93.5 ab	93.9 a
	50	98.2 a	98.4 a	97.0 a

* - Cultures were grown on media ammended with each fungicide at selected concentrations.

Fig. 00.

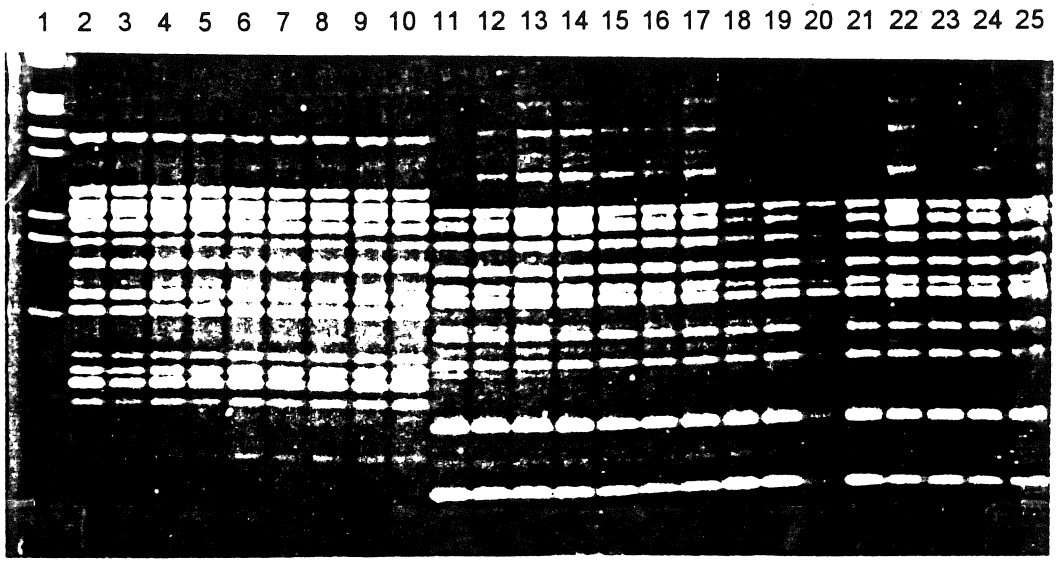
A. Spur Infections - Note Fruit Infections and Flagging of Leaves

B. Close-Up of Spur Infection Arrow indicates infected Fruit.



C. Anthracnose of Almond Fruitlets Showing Orangish Sunken Lesions. Arrows indicate sectioned fruitlet showing decay of the kernel.

Fig. 0. Eletrophoretic separation of PCR amplification products using a random primer for twenty-four isolates of *Colletotrichum acutatum.* Two distinct banding patterns (lanes 2-10 and 11-25) are evident representing the two sub-populations.



* - Each lane represents DNA from a different almond isolate of *C. acutatum.*

Fig. 1. In vitro, fungicide disk assays.

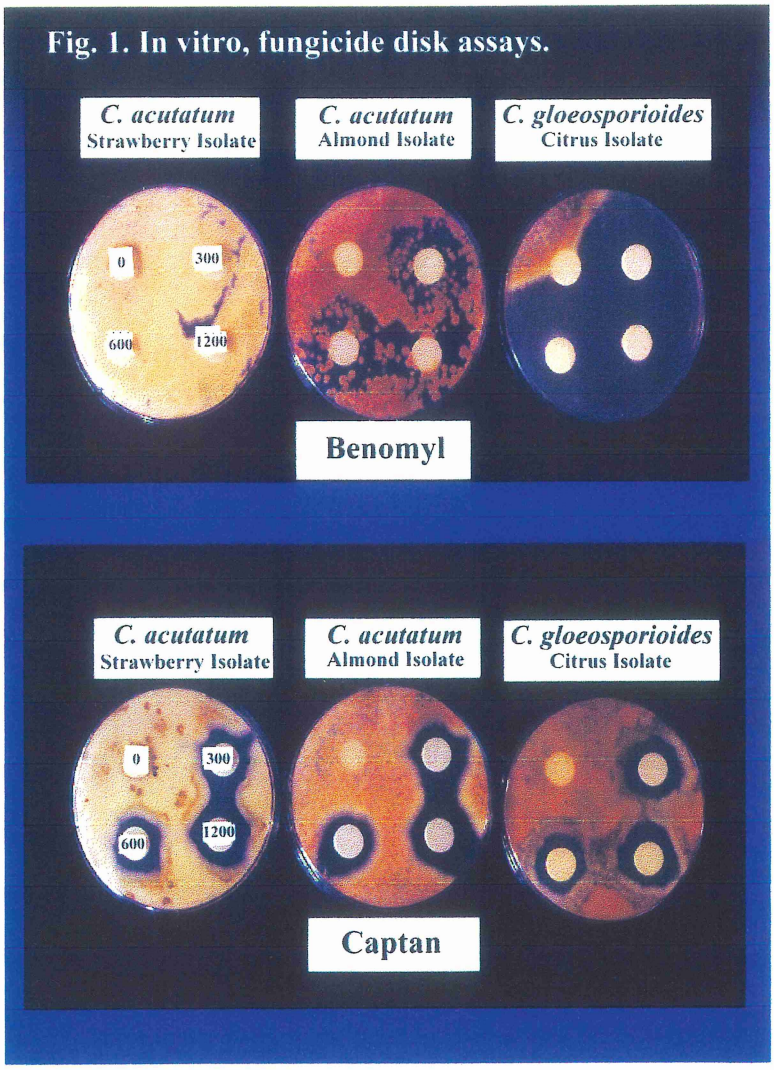
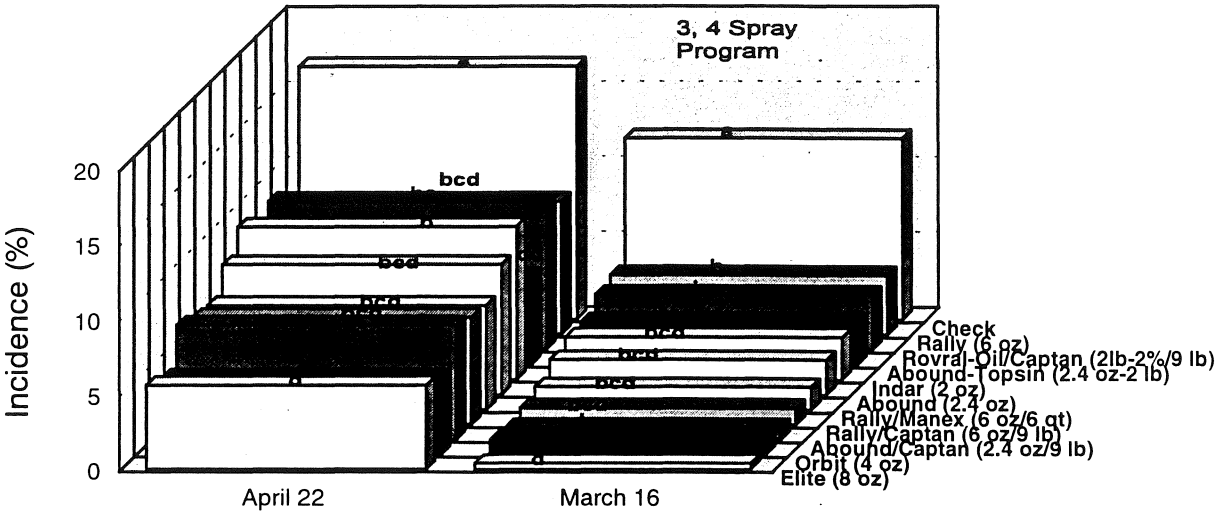


Fig. 2.

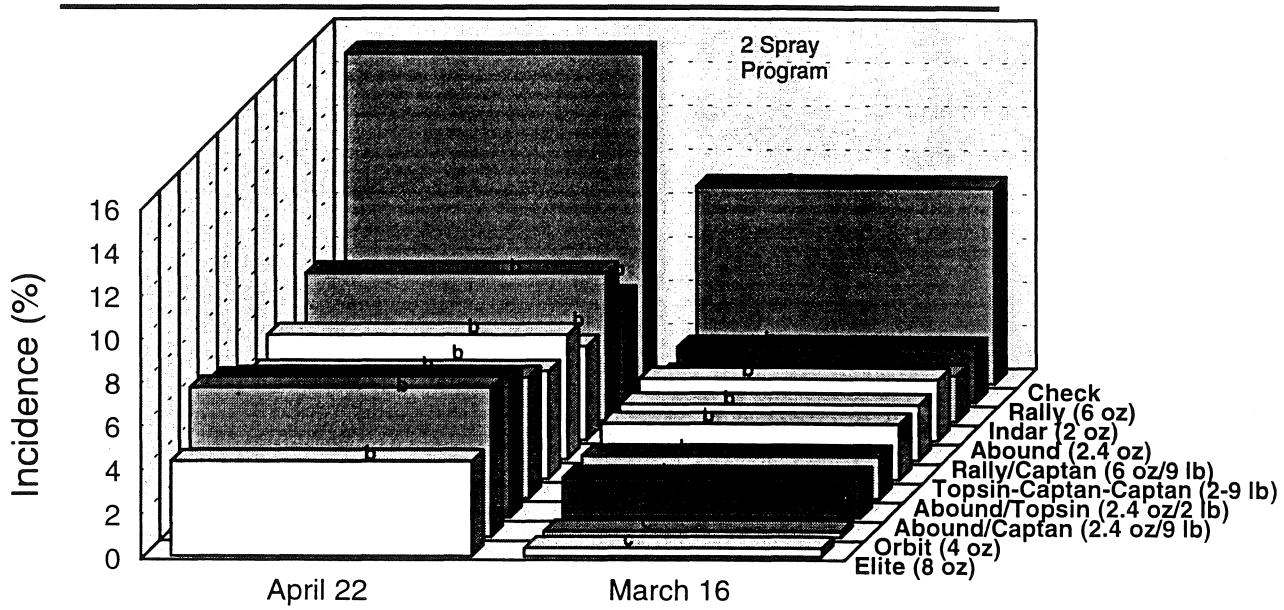
Efficacy of fungicide treatments for control of anthracnose of Carmel almonds in Merced Co. - Spring 1996



Treatments were applied using an air-blast sprayer calibrated for 100 gal/A. Timing was based on phenological stage: Pink bud, full bloom, and 1-, & 2-wk petal fall.

Fig. 3.

Efficacy of fungicide treatments for control of anthracnose of Price almonds in Butte Co. - Spring 1996



Treatments were applied using an air-blast sprayer calibrated for 100 gal/A. Timing was based on phenological stage: Full bloom and Petal Fall.