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*Prepared for the Almond Board of California*

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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

Research on almond anthracnose continued in 1997. The fungal pathogen previously was characterized and identified as *Colletotrichum acutatum*. Using molecular and cultural techniques, two distinct sub-populations could be identified among the isolates of *C. acutatum* collected from almond that correlated with cultural pigmentation (pink vs. gray), spore morphology, virulence, and sensitivity to benomyl at low conidial concentrations. In 1997, the disease was also found to cause postharvest problems on the 1996 crop. The pathogen was isolated from processed almond kernels in storage. Symptoms included brown and blue to purplish discolorations of the kernel tissue similar to concealed kernel damage. Percentage of quality assurance tests with positive samples of anthracnose-infected kernels was 0, 2, 10, and 10% for Nonpareil, Butte, Carmel, and Mission, respectively. Additionally, preliminary studies were conducted to determine the cause of dieback symptoms in trees associated with almond anthracnose. In laboratory bioassays using tomato seedlings or detached peach and almond shoots, cultural filtrates of the fungus were toxic to leaf tissue causing symptoms similar to those observed in the field. Thus, the disease can cause direct pre- and postharvest losses of the almond crop by infecting fruit and kernels, as well as potentially indirect losses by causing shoot dieback of trees. In laboratory and field trials in Fresno and San Joaquin Co., varietal comparisons of Butte, Carmel, Fritz, Harvey, Mission, Monterey, Peerless, Price, Thompson, and NePlus Ultra as well as Nonpareil using natural incidence and fruit inoculation studies indicated that all varieties are susceptible to *C. acutatum* isolates from almond. *C. gloesporioides* from citrus was non-pathogenic or caused only small lesions. Propiconazole, tebuconazole and fluazinam were the most effective compounds in laboratory assays for inhibiting mycelial growth, whereas captan, chlorothalonil, and fluazinam were most effective in inhibiting spore germination. Fungicides evaluated in field trials included: azoxystrobin, fenbuconazole, myclobutanil, propiconazole, tebuconazole, iprodione, captan, chlorothalonil, and thiophanate-methyl. Dry spring conditions prevented efficacy data from

being obtained. A Section 18, emergency registration petition, for propiconazole (Break 45WP) was re-submitted to EPA for the 1998 season.

## INTRODUCTION

In recent years, almond anthracnose has become a widespread and serious problem in the major almond growing regions in California. Substantial economic losses occurred in 1995 and 1996. Symptoms of the disease occur on blossoms, developing and maturing fruit, and on spurs and branches with often extensive leaf yellowing. Throughout the spring and summer, if conducive conditions persist, infections develop on mature fruit, and branch dieback becomes apparent as the fungus advances into woody tissue.

The causal organism has been characterized using conidial morphology, temperature relationships, molecular techniques, and fungicide sensitivity tests and was identified to be *Colletotrichum acutatum* (Adaskaveg & Hartin, 1997). Using molecular techniques that detect DNA polymorphisms we previously identified two distinct sub-populations (A and B) that sometimes were found in the same orchard. These two sub-populations also differed in their cultural pigmentation and in their sensitivity to the fungicide Benlate in a filter paper disk assay. The epidemiological significance of these sub-populations remained unknown and was a subject of our 1997 research. Additional research objectives in 1997 included a disease survey during the growing season, the evaluation of almond varieties for anthracnose resistance and field and *in vitro* evaluation of fungicides for the development of improved management strategies. Postharvest kernel damage which was reported for the first time in 1997 was also investigated.

## OBJECTIVES

I. Epidemiology
- A. Continue to survey isolates from major almond growing regions within California
- B. Determination of overwintering mechanisms
- C. Evaluation of host susceptibility

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. Fungicide efficacy studies
- C. Orchard sanitation and anthracnose management

## MATERIALS AND METHODS

***Fungal isolation and culturing.*** 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. Conidial morphology was studied in cultures grown on wheat straw agar.

***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 an UV transilluminator.

***Pathogenicity assays.*** Almond fruit (variety Butte) were wound-inoculated (1 x 1 x 2 mm wound) using 20 ml of an aqueous suspension (2 x $10^5$ conidia/ml) of *Colletotrichum* spores and incubated in a plastic container for 14 days at 20 C, >95% relative humidity.

***Evaluation of the* in vitro *production of phytotoxic compounds by the almond anthracnose pathogen.*** A *Colletotrichum acutatum* isolate representing sub-population A was grown in modified Richard's solution (Goodman, 1959). 250 ml Erlenmeyer flasks containing 100 ml medium were inoculated with agar plugs containing mycelium and conidia and incubated on a shaker at 200 rpm at room temperature. After two weeks the culture filtrate was obtained by filtering twice through filter paper (Eaton-Dikeman grade 515). This crude culture filtrate was kept frozen until it was used for experiments. Aliquots were then passed through a 0.45 mm filter to eliminate any viable conidia and diluted 1:1 to1:4 with sterile distilled water. For the bioassay, 3 wk old tomato seedlings or peach or almond twig cuttings were incubated in plastic vials with 20 ml of test solution under laboratory conditions with continuous light.

***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. In the fungicide-treated almond fruit assay almond fruit (var. Butte) was surface-sterilized for 1 min with 400 ppm bleach, air-dried, wounded (1 x 1 x 2 mm wound), sprayed with fungicides (150 ppm) and air-dried again. 20 ml of spore suspension of almond anthracnose genotype A (2 x $10^5$ spores/ml) was then pipetted into each wound and the fruit was incubated in a moist chamber at 20 C. The outside and inside lesion diameters were measured after 2 weeks. Fungicides evaluated during 1997 included: captan (Captan 50WP), chlorothalonil (Bravo), and fluazinam ( ), benomyl (Benlate 50WDG), propiconazole (Orbit 45WP), tebuconazole (Elite 45DF), For these compounds and compounds that were evaluated previously, e.g.,

myclobutanil (Rally 40WP), propiconazole (Break 45WP), tebuconazole (Elite 45DF), and benomyl (Benlate 50WDG) $EC_{50}$ were determined.

***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. Epidemiology

***Disease survey during the 1997 growing season and characterization of field isolates.*** Five orchards that were heavily infected in 1996 were re-sampled for anthracnose in 1997. Isolates of *Colletotrichum acutatum* could be recovered from all five orchards. Using molecular techniques and comparing cultural morphologies both sub-populations were identified among 16 isolates. Therefore, the pathogen was present in the field although the unusual dry spring weather conditions in 1997 were not conducive.

***Pathogenicity assays to clarify the epidemiological role of the two anthracnose sub-populations.*** Almond fruit was wound-inoculated in the laboratory with representatives of the two California anthracnose sub-populations and lesion sizes were recorded after a two week incubation period. The results indicated that the two genotypes differed significantly in their virulence (Table 1). Lesion sizes after inoculation with sub-population B were much reduced as compared to sub-population A, however, disease symptoms were identical, both genotypes producing circular orangish to tan lesions. As isolations from naturally field-infected tissues demonstrated, both genotypes are able to cause severe damage in the field. Field inoculation studies will have to be carried out to evaluate the epidemiological impact of the two anthracnose sub-populations in more detail. Of the other *Colletotrichum* isolates that were included into the experiment, an isolate from peach also was very virulent, producing lesions of the same size as almond genotype A (Table 1). These two groups of isolates also exhibited identical DNA fingerprint patterns when using four different primers in PCR amplifications. Two molecularly distinct isolates of *C. acutatum* from strawberry caused significantly different lesion sizes, one of the isolates being quite virulent while the other one produced only very small lesions, not exceeding the wounding site, similar to *C. gloeosporioides*.

Table 1. Pathogenicity of isolates of *Colletotrichum* species in laboatory wound-inoculation studies on almond fruit.

| *Colletotrichum* species/Host | Lesion diameter (mm) |
|---|---|
| Almond genotype A | 12.73 a |
| Almond genotype B | 3.94 c |
| *C. acutatum*/peach | 12.21 a |
| *C. acutatum*/strawberry 1 | 7.56 b |
| *C. acutatum*/strawberry 2 | 0.85 d |
| *C. gloeosporioides*/citrus | 1.90 cd |

* - Values followed by the same letters are not statistically different ($P > 0.05$) based on analysis of variance and least significant difference mean separation.

***Determination of overwintering mechanisms.*** In February of 1997 isolations were carried out from fruit mummies collected in four orchards. *Colletotrichum acutatum* could be recovered from hull, shell, and endosperm tissue. The recovery rate in two orchards (based on the number of mummies investigated) was 100 %. This indicates that overwintering fruit mummies are inoculum sources for early spring infection caused by rain splash dispersal of spores.

***Evaluation of almond varieties for anthracnose resistance.*** Almond fruit of the varieties Nonpareil, Carmel, Fritz, Butte, Peerless, Price, Sonora, Mission, Padre, Neplus, Thompson, and Wood Colony were inoculated in the field at two different locations or in the laboratory and lesion sizes were evaluated. As in our brown rot host resistance studies, variation occurred between the two field sites indicating that environmental factors, cultural practices, and tree age may effect results. Regardless, the varieties Butte and Peerless were very susceptible at both locations (Fig. 1). Other varieties, including Fritz, Price, NePlus Ultra, Sonora, Padre and Wood Colony, as well as Nonpareil, varied in their susceptibility depending on the site. All varieties were susceptible to *C. acutatum* isolates from almond. *C. gloesporioides* from citrus was non-pathogenic or caused only small lesions. Results from laboratory inoculations These experiments need to be repeated in 1998.

***Physiological mechanisms possibly involved in branch dieback and leaf yellowing.*** Whereas isolations of the pathogen from infected fruit usually were highly successful, recovery rates from wood and leaf tissue were only 0-10 %. This observation suggested an indirect way of disease symptom production, e.g. not by the presence of the pathogen at the site of symptom expression. Since phytotoxic compounds have been described from species of *Colletotrichum* (Amusa, 1994; Frantzen *et al.*, 1982; Goodman, 1959; Ohra *et al.*, 1995) we tested this hypothesis for the almond anthracnose pathogen. In preliminary studies, incubation of peach and almond cuttings or tomato seedlings in culture filtrates of the fungus (minus the fungus) resulted in wilting and leaf necrosis after 2-3 days (Table 2). The initial leaf symptoms on almond with water-soaked spots were very similar

to those often observed in the field. Compounds in the cultural filtrate may be responsible for disease symptom development on leaves and branches. Our goal is to isolate and chemically characterize possible phytotoxic metabolites.

Table 2. Effect of culture filtrates of *C. acutatum* from almond compared to growth medium or water on tomato plants and almond cuttings.*

| Symptoms | Culture filtrate | Growth medium | Water |
|---|---|---|---|
| Tomato leaf lesions | + | - | - |
| Tomato plant wilting | + | - | - |
| Almond leaf lesions | + | - | - |
| Almond leaf wilting | + | - | - |

* - Plants were incubated in each solution for 2-3 days.

***Postharvest damage of almond kernels in storage.*** Quality concerns of almonds in storage prior to further processing led to our initial postharvest studies of almond. Symptoms on the kernels included brown and blue to purplish discolorations when cut open. The anthracnose pathogen was isolated from almond kernels harvested in 1996 and kept in storage into 1997. Percentage of quality assurance tests with one or more kernels infected was 0, 2.1, 9.8, and 10 % for Nonpareil, Butte, Carmel, and Mission, respectively (Table 3). Total infection of almond kernels was estimated to be less than 0.02%. Isolations from blanched fruit were negative, indicating that heat treatment during the blanching process probably killed the fungus. Additional isolations were carried out from five almond lots. *Colletotrichum* was mainly isolated from kernels that showed discolorations when cut open (50% of the isolations were successful). Isolations from shriveled kernels or kernels with gumming were much less successful, and only 8.5% or 4.3% of the kernels yielded *Colletotrichum*, respectively. From none of the healthy appearing kernels could *Colletotrichum* be isolated. *Rhizopus* sp. and *Aspergillus* sp. were isolated with a high frequency (46% and 36.6%, respectively) from all kernel categories. Our goal is to define the conditions that lead to postharvest kernel damage caused by *C. acutatum* and we already initiated studies to evaluate the effect of storage humidity and kernel moisture content on disease development.

Table 3. Postharvest anthracnose damage in processed almond kernels caused by *C. acutatum.*

| Variety | Total Tests | No. of tests with Anthracnose infected kernels* | Tests with anthracnose infected kernels (%) |
|---|---|---|---|
| Nonpareil | 38 | 0 | 0 |
| Butte | 241 | 5 | 2.1 |
| Carmel | 133 | 13 | 9.8 |
| Mission | 420 | 42 | 10 |

* - Tests with one or more anthracnose-discolored kernels in a 0.72 kg sample.

## II. Identification of *Colletotrichum* sp.

***Cultural and molecular characterization.*** As already indicated above, both sub-populations of anthracnose were identified among the 1997 isolations using the molecular techniques that we developed previously. We conducted a careful comparison of conidium morphology between isolates of the two sub-populations that were grown on straw agar. The conidia of the sub-populations with pink colony pigmentation (sub-population A) were clearly pointed at each end, whereas those of the gray cultures (sub-population B) were less pointed, however, were very distinct from spores of *C. gloeosporioides* with their broadly rounded ends.

## III. Preliminary management strategies

**In vitro *evaluation of additional fungicides.*** Alternative chemical control compounds in addition to the currently registered fungicides need to be identified in order to design rotation programs using fungicides with different modes of action. These rotation programs are more likely to reduce the potential of development of resistant pathogen populations. We re-evaluated the *in vitro* effect of chlorothalonil (Bravo) on spore germination and mycelial growth of *Colletotrichum acutatum* from almond and studied the effect of another compound, Fluazinam. Captan was included as a positive control in these experiments. Spore germination was completely inhibited by chlorothalonil or Fluazinam at concentrations of 0.5 ppm or higher, whereas concentrations of 5 ppm or higher were required for Captan. Chlorothalonil had no effect on mycelial growth at concentrations below 50 ppm. Mycelial growth was inhibited by 50% using 100 ppm Captan or 0.5 ppm Fluazinam. $EC_{50}$ values for inhibition of mycelial growth and spore germination were calculated for fungicides evaluated during 1997 and previously (Table 4).

Table 4. $EC_{50}$ values of selected fungicides evaluated in in vivo mycelial growth or spore germination studies.

| Fungus | Fungicide | EC50 (ug/ml) |
|---|---|---|
| *C.acutatum* 23-1 | captan | 180 |
| *C.acutatum* 23-1 | benomyl | ? |
| *C.acutatum* 23-1 | tebuconazole | 0.2 |
| *C.acutatum* 23-1 | propiconazole | 0.6 |
| *C.acutatum* 23-1 | myclobutanil | 1 |
| | | |
| *C.acutatum* 24-1 | captan | 160 |
| *C.acutatum* 24-1 | benomyl | 1.6 |
| *C.acutatum* 24-1 | tebuconazole | 0.22 |
| *C.acutatum* 24-1 | propiconazole | 0.28 |
| *C.acutatum* 24-1 | myclobutanil | 1.6 |
| *C.acutatum* 24-1 | chlorothalonil | >100 |
| *C.acutatum* 24-1 | fluazinam | 0.6 |

* - $EC_{50}$ values were obtained by transferring spore dilutions agar plugs containing mycelium of the fungus to fungicide ammended agar medium. Values were calculated based on regression analysis of percent

reduction of growth or spore germination on log concentration of the selected fungicide. All models were significant with $R^2$ values greater than 0.80 except for benomyl due to variable sensitivity.

When detached almond fruit was first wounded, then sprayed with fungicides and subsequently inoculated with a spore suspension of *C. acutatum*, tebuconazole completely inhibited lesion formation (Table 5). After treatments with captan or propiconazole only very limited lesion development was observed. Lesion sizes after benomyl treatment did not differ from the untreated control fruit.

Table 5. Efficacy of fungicides against C. acutatum on fungicide-treated, detached almonds.

| Fungicide | Outer lesion diameter (mm) |
|---|---|
| Check | 13.47 a |
| Benomyl | 11.49 a |
| Chlorothalonil | 7.29 b |
| Propiconazole | 3.24 c |
| Captan | 2.81 c |
| Tebuconazole | 0 c |

* - Almond fruit (var. Butte) were surface sterlized, wounded, sprayed with fungicides (150 *ug*/ml) and inoculated with 20 ul of a spore suspension of C. acutatum (genotype A, at 20K spores/ml) and incubated for 14 days.

** - Values followed by the same letters are not statistically different ($P > 0.05$) based on analysis of variance and least significant difference mean separation.

***Field studies.*** Due to the dry spring in the 1997 season with very low rainfall accumulations throughout the almond growing regions of the state, anthracnose incidence was very low. In both our plots in Butte and Merced Co., disease incidence in the non-treated trees was less than 1%. Thus, no efficacy data was obtained in our comparisons of eight different fungicide treatments. These plots will be re-established for the 1998 season.

## REFERENCES

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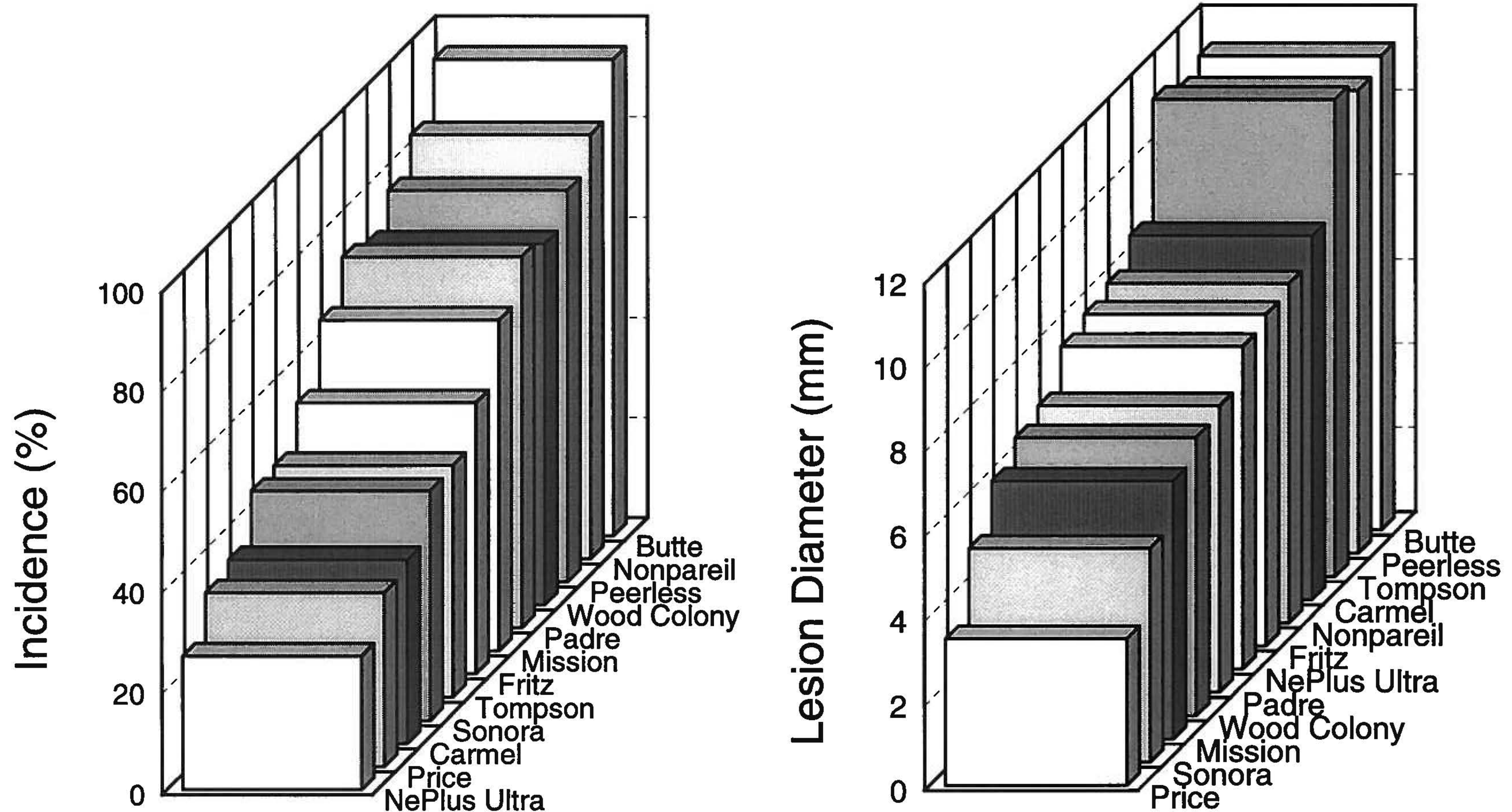
# Fig 1: Susceptibility of Selected Almond Cultivars to Anthracnose In Wound Inoculated Fruit Studies

## - Field Studies 1997 -




Immature fruit were wound-inoculated with *C. acutatum* Genotype A using a conidial suspension (30K spores/ml). Values are the average of two trials conducted in San Joaquin and Fresno counties.