

## **Relationship between Fertilization and Pistachio Diseases**

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### **Objectives:**

1. Determine the effects of fertilization on pistachio diseases (*Botryosphaeria* and *Alternaria* blights) in a greenhouse, and
2. Determine the effects of fertilization on pistachio disease resistance/susceptibility compounds.

### **Summary**

Three levels (75%, 100%, and 200%) of nitrogen (N), phosphorous (P), and potassium (K) elements were established by fertilizing potted trees with modified Hoagland solution (1 liter per plant per week). The 100% solution contained 0.21 g N, 0.032 g P, and 0.234 g K and various microelements per liter solution. Potted pistachio trees were inoculated with a 20,000 mycelial fragments/ml

suspension of *B. dothidea* in a greenhouse at Kearney Agricultural Center. Disease index was evaluated 15 days after inoculation. The 200% K rate significantly reduced the severity of Botryosphaeria blight on pistachio leaves as compared with normal fertilization (100% N, P, and K). In 2000, potted, 2- and 1-year-old trees were obtained from a pistachio nursery for experiments I and II, respectively. Three levels of nitrogen (N), phosphorous (P) and potassium (K) elements were established by feeding 4-replicated potted trees with modified Hoagland solution once per 2 weeks (1 liter per tree). Three levels of calcium were established by spraying 4-replicated trees with 0.1%, 0.2% and 0.4%  $\text{CaCl}_2$  and  $\text{Ca}(\text{NO}_3)_2$  (50ml per tree) in experiment I and II, respectively. After fertilizing the trees for three times, all trees were sprayed with 20,000 of mycelial fragments/ml suspension of *B. dothidea*. To create conditions favorable for infection, the inoculated trees were covered with a plastic bag for 12 hours. The disease severity was assessed as described above 30 days after inoculation. Analysis of variance for DIs affected by nutritional stress was conducted using ANOVA of SAS. In 2000 experiment I, although there were no significant differences in DIs ( $F = 1.82$ ,  $P = 0.1193$ ) among the various treatments, the DI of 200% K treatment was reduced by 30% as compared to that of the control treatment (100% each of N, P, and K). In this experiment, 2% and 4%  $\text{CaCl}_2$  showed to be phytotoxic to pistachio, thus in experiment II,  $\text{Ca}(\text{NO}_3)_2$  was used instead of  $\text{CaCl}_2$ . In experiment II, the DI of 200% K treatment was reduced significantly ( $P < 0.05$ ) by 27.3% as compared to that of the control treatment, and the DIs of the trees sprayed with 0.1%, 0.2%, and 0.4%  $\text{Ca}(\text{NO}_3)_2$  were reduced significantly ( $P < 0.05$ ) by 33%, 24% and 19%, as compared to that of the control treatment, respectively. The general conclusion from these trials was that fertilizing pistachio trees with high level of potassium or spraying trees with calcium nitrate reduce the severity of Botryosphaeria blight.

Similar to the Botryosphaeria blight experiments, the susceptible pistachio cultivar Kerman was selected for a greenhouse experiment to study the effect of fertilization on Alternaria late blight. Three levels (50%, 100%, and 200%) of nitrogen (N), phosphorous (P), and potassium (K), and three levels, 0.1%, 0.2%, and 0.4% of  $\text{Ca}(\text{NO}_3)_2$  were established in 4 each replicated potted trees per treatment. The 100% solution contained 0.21 g N, 0.032 g P, and 0.234 g K and microelements per liter solution. One liter was supplied per plant per week. After fertilizing the trees for 2 months, all trees were spray-inoculated with a 20,000/ml spore suspension of *Alternaria alternata*. Plants were checked for infection (leaf lesions) and evaluated 30 days after infection. Unfortunately, no infections developed by *A. alternata* during the time when this experiment was done (in June). Another set of trees were inoculated in August as described above with *A. Alternaria* spore suspension and recorded for disease 30 days after inoculation. Because symptoms did not developed, latent infections on leaves were recorded using the overnight freezing incubation technique (ONFIT). The ONFIT results from the greenhouse experiments showed that the severity of Alternaria late blight on pistachio was not affected by applying potassium nitrate or potassium chlorite. However, the disease was reduced significantly by eight sprays of 100

mM of nitrogen, applied either as  $\text{NH}_4\text{NO}_3$  or  $\text{Ca}(\text{NO}_3)_2$ . Calcium chlorite at 50 mM or potassium nitrate at 100 mM rates showed trends towards reducing the number of latent infections of *A. alternata* per leaf of pistachio as compared to the control trees. But eight applications of  $\text{CaCl}_2$  at 50 mM or KCl at 100 mM rates caused phytotoxicity to 1-year-old pistachio trees in these experiments.

The general conclusion from these trials is that fertilizing trees with high levels of potassium or spraying trees with calcium nitrate can reduce the severity of Botryosphaeria blight; also fertilizing pistachio trees with ammonium nitrate or calcium nitrate can reduce Alternaria late blight.

### Work Description:

#### Determination of fertilization effects on pistachio diseases (Botryosphaeria and Alternaria blights) in a greenhouse.

All subtasks (1 through 9) of this task have been completed. However, because of undetermined reasons, inoculations with *Alternaria* suspensions were not successful in the first inoculation experiment. In the first year of the study, the plants were inoculated only with *B. dothidea*, and emphasis was given on Botryosphaeria blight disease. In the second year, one set of pistachio trees were again inoculated with *B. dothidea* and a second set of trees were inoculated with *A. alternata*. Results are presented for each disease separately.

*Inoculations with B. dothidea.* The susceptible pistachio cultivar Kerman to Botryosphaeria blight was selected for greenhouse experiments. Potted, 2-year-old trees were obtained from a pistachio nursery for experiments in 1999 and experiment I in 2000. One-year-old trees were used in experiment II in 2000. Three levels (75%, 100%, and 200% in 1999 and 50%, 100%, and 200% in 2000) of nitrogen (N), phosphorous (P) and potassium (K) elements were established by feeding 4-replicated potted trees with modified Hoagland solution (Hoagland, 1950) once per 2 weeks (1 liter per tree). Three levels of calcium were established by spraying 4-replicated trees with 0.1%, 0.2% and 0.4%  $\text{CaCl}_2$  and  $\text{Ca}(\text{NO}_3)_2$  (50ml per tree) for experiment I and II in 2000, respectively. After fertilizing the trees for 2 months, all trees were sprayed with 20,000 of spores /ml suspension of *B. dothidea*. To create conditions favorable for infection, the inoculated trees were covered with a plastic bag for 12 hours. The disease severity was assessed 15 days after inoculation. The following system was used for severity assessment: 0 = leaves without lesions, 1= lesion area less than one quarter of the leaf area, 2 = lesion area between one quarter and half of the leaf area, 3 = lesion area between half and three quarters of the leaf area, and 4 = lesion area greater than three quarters of the leaf area. The disease index (DI) for each tree was calculated using the formula:

$$DI = \left( \sum_{i=0}^4 N_i * i \right) / \sum_{i=0}^4 N_i$$

Where  $i$  is severity (0 to 4) and  $N_i$  is the number of leaves with the severity of  $i$ . Analysis of variance for DIs of pistachio trees affected by different levels of fertilization was conducted using ANOVA of SAS.

*Inoculations with Alternaria alternata.* Similar to the Botryosphaeria blight experiments, the susceptible pistachio cultivar Kerman was selected for a greenhouse experiment to study the effect of fertilization on Alternaria late blight. The first inoculations early in the season did not develop any symptoms. Because Alternaria late blight requires senescing leaves in order to infect, the experiment was performed again in August 2001 when leaves were fully mature and started senescing. Potted, 1-year-old Kerman pistachio trees were obtained from a nursery for the greenhouse experiments. Two levels of nitrogen (N) (25 and 100 mM), potassium (K) (25 and 100 mM), and Calcium (Ca) (12.5 and 50 mM) were established by spraying six replicated potted trees with  $\text{NH}_4\text{NO}_3$ , KCl,  $\text{KNO}_3$ ,  $\text{CaCl}_2$ , or  $\text{Ca}(\text{NO}_3)_2$ . After fertilizing the trees once a week for 2 months, all trees were sprayed with 20,000 spores/ml suspension of *A. alternata*. To create conditions of high humidity favorable for infection, the inoculated trees were covered with a plastic bag for 12 hours. Two weeks after inoculation, leaf samples were collected from each tree and analyzed for latent infections using the OverNight Freezing Incubation Technique (ONFIT; a protocol is attached). Analysis of variance for latent infections (expressed as number of lesions per leaf) by *A. alternata* on pistachio leaves among various treatments was conducted using ANOVA of SAS. All subtasks (1 through 9) of this task for *Alternaria* inoculations have been completed.

### **Determination of fertilization effects on pistachio disease resistance/susceptibility compounds.**

A 10 g of pistachio leaves for each tree was harvested, snap frozen in liquid nitrogen, and stored at  $-20^\circ\text{C}$  for determination of resistant compounds [phenylalanine ammonia lyase (PAL), peroxidase (POX),  $\beta$ -1,3-glucanase, chitinase, and phenolic compounds]. When we decided to determine disease resistance/susceptibility compounds in the frozen pistachio leaves, we encountered two major problems. 1) When we tried to use our existing UV-spectrophotometer that has not been used for a long time to measure disease resistance/susceptibility compounds, we discovered that it was out of order and worthless of fixing. The manufacturer told us that it would be as expensive to fix it as to buy a new upgraded spectrophotometer. In the original proposal to CDFA/FREP, we did not request any funds for equipment. Thus, we had to wait about 8 months until we gathered some various donors funds in order to be able to buy a new spectrophotometer [SPECTRO-UV-VIS RS (Labomed, Inc., Culver City, CA)]. The second and most serious problem was that in 2001 our Center experienced numerous power blackouts. In one of these our  $-20^\circ\text{C}$  refrigerator did not kick back to its normal temperature, which resulted in destruction of our frozen leaf samples. It is unfortunate that we were not able to analyze the

samples we collected from the various treatments of several experiments from both *Botryosphaeria* and *Alternaria* inoculations.

We decided though to include in this report the results from a related experiment in which we were able to measure PAL activity in various cultivars of pistachio inoculated with *B. dothidea* suspensions using our new UV-spectrophotometer. PAL is the key enzyme in the phenylpropanoid biosynthesis pathway. PAL activity is frequently increased in plants infected by pathogens, and the levels of PAL activity are often correlated with disease resistance. Enhanced PAL activity is very often associated with resistance phenomena such as lignin production and phenol accumulation. A high correlation was found in noninfected tomato susceptible or resistant to *Verticillium dahliae*, muskmelon to *Sphaerotheca fuliginea* and *Pseudoperonospora cubensis*, and lettuce to *Bremia lactucae*.

**Determination of phenylalanine ammonia lyase (PAL) activity.** In this study, one gram of leaves for each cultivar was collected before inoculation and 3 days after inoculation, respectively. The sampled leaves were instantly frozen in liquid nitrogen, and kept at -70°C for determination of PAL activity. PAL activity was measured by the procedure described by Redman (1999). One gram of sample was ground in liquid nitrogen, and placed into 5 ml of 0.01 M sodium phosphate buffer (pH 6.0). After the samples were centrifuged (10,000 g for 5 min at 4°C), a 50 µl of the above supernatant was added into 1.95 ml reaction mixture consisted of 6 µM L-Phe and 0.05 M Tris-HCl buffer (pH 8.0). After 60 min at 37°C, the reaction was terminated by the addition of 0.05 ml of 5 N HCl. The reaction terminated by HCl before incubation was used as control. PAL activity was expressed in absorbance at 290 nm per 100 µg of fresh leaf weight. The experiment was repeated six times and data were analyzed using ANOVA of SAS.

## Results, Discussion, and Conclusions:

***Botryosphaeria blight.*** In 1999, there were no significant differences among the percentages of infected leaves among the various treatments. However, the 200% K treatment significantly reduced severity of *Botryosphaeria* blight on pistachio leaves as compared with the normal fertilization (100% N, P, and K) (Table 1 and Fig. 1). Results from the fertilization experiment in 1999 showed that nutritional stress did not increase the incidence of *Botryosphaeria* disease as compared with the normal nutrition level (no stress). This could be because of enough N, P, or K was stored in the potted trees in the first year or not enough nutritional stress was established by applying the treatments in this experiment. However, increasing K fertilization may have a significant effect in reducing disease in pistachios.

Potassium is an essential plant nutrient and is involved in most plant functions. Potassium fertilization affects not only the yield and quality of crops but also

levels of plant diseases. Zeng et al. (1997 & 1999) reported that application of potassium reduced the percentages of blank and stained pistachio nuts caused by *B. dothidea* or *Alternaria alternata*. Our data also showed that high levels of potassium significantly reduced the severity of *Botryosphaeria* blight in greenhouse pistachios. Potassium deficiency has been identified in many California pistachio orchards (Brown et al., 1995), primarily due to: a) depletion of soil K after long term cultivation with limited use of K fertilizers; b) increased crop yield, giving rise to higher K demand and removal by pistachio trees; c) strong K-fixation capacity of soils in many orchards, reducing soil K availability and utilization efficiency of applied K; and d) increased use of nitrogen fertilizers, particularly the ammonium-N which tends to acidify the soil and inhibit K uptake by plants (Chung & Zasoski, 1993). Thus, abundant K deficiency in pistachio orchards may have contributed partially to the *Botryosphaeria* blight outbreaks throughout California pistachio orchards.

For experiment I in 2000, although there were no significant differences ( $F = 1.82$ ,  $P = 0.1193$ ) (Table 2) in disease indices (DIs) among the various treatments, the DI for the 200% K treatment was reduced by 30% as compared to that of the control treatment (100% each of N, P, and K). In this experiment, 2% and 4%  $\text{CaCl}_2$  were phytotoxic to pistachio, thus,  $\text{Ca}(\text{NO}_3)_2$  was used instead of  $\text{CaCl}_2$  in experiment II in 2000. In experiment II, the DI of 200% K treatment was reduced significantly ( $P < 0.05$ ) by 27% as compared to that of the control treatment, and the DIs of the trees sprayed with 0.1%, 0.2%, and 0.4%  $\text{Ca}(\text{NO}_3)_2$  were reduced significantly ( $P < 0.05$ ) by 33%, 24%, and 19%, as compared to that of the control treatment, respectively (Table 2).

The general conclusion from the 2 years (1999/2000 and 2000/2001) experiments is that there were no significant differences among the percentages of infected leaves and disease indices caused by *Botryosphaeria dothidea* among the various treatments of N and P fertilization. However, only the 200% K rate treatment significantly reduced severity of *Botryosphaeria* blight by about 30% on pistachio leaves as compared with the normal (100% N, P, and K) fertilization treatments (Tables 1 and 2 and Fig. 1).

In a separate experiment as described in the work description section, stems of fertilized and non-fertilized trees were inoculated with a mycelial plug placed in a wound created by a cork borer. Results of the stem inoculation experiment were variable. A partial reason of this variability might be differences in the diameter of stems. Generally, lesions were longer in shoots with smaller diameter than in those with large diameter.

***Alternaria late blight.*** The results from greenhouse experiments showed that the severity of *Alternaria* late blight on pistachio was reduced significantly by eight sprays of 100 mM of nitrogen (either  $\text{NH}_4\text{NO}_3$ , or  $\text{Ca}(\text{NO}_3)_2$ ) (Fig. 2) Eight applications of 50 mM  $\text{CaCl}_2$  or 100 mM KCl were phytotoxic to 1-year-old pistachio trees in the greenhouse experiments.

Our data also showed that sprays of calcium might reduce the severity of Botryosphaeria blight in pistachio and also applying N as  $\text{Ca}(\text{NO}_3)_2$  (high rate) reduced Alternaria late blight (Fig. 2). These results are in agreement with previously published reports on other hosts. Fertilizing with calcium reduced *Fusarium* wilt of tomato (Corden 1965) and muskmelon (Spiegel et al. 1987), protected vinca seedling against infection of *Phytophthora parasitica* (Von Broembsen, et al 1997), and also increased resistance of bean hypocotyls to *Rhizoctonia solani* (Bateman, et al. 1965) and soybean to *Colletotrichum dematium* (Muchovej, et al. 1980). Liming of low calcic soils caused a reduction of tomato gray mold incidence (Stall, 1963). Volpin et al (1991) have also reported a similar effect of calcium in reducing the susceptibility of rose flower to gray mold caused by *Botrytis cinerea*.

Many plant pathogens, such as *Botryosphaeria ribis* (Bateman, 1963), *Rhizoctonia solani* (McClendon, 1960), and *Erwinia carotovora* (McGuire, et al., 1986), *Monilinia fructicola* (Biggs, et al. 1997), *Penicillium expansum* (Conway, et al. 1988), produce pectic enzymes to destroy various components of host plant cell walls. However, the activities of these pectic enzymes were greatly inhibited by  $\text{Ca}^{++}$  or  $\text{Ba}^{++}$ , less inhibited by  $\text{Mg}^{++}$ , and not significantly influenced by certain monovalent ions (Bateman, 1964, Corden, 1965). Therefore, the effects of calcium on Botryosphaeria blight of pistachio might be related to the inhibition of calcium against polygalacturonase and other pectic enzymes produced by *B. dothidea*.

### **Determination of fertilization effects on pistachio disease**

**resistance/susceptibility compounds:** Because of the destruction of the pistachio leaves saved from the trees of the various treatments, analyses for disease resistance/susceptibility compounds were not performed.

**Activity of phenylalanine ammonia lyase (PAL).** The PAL activities of six tested cultivars showed significantly different both before and after inoculation (Fig. 3). Unfortunately, there was not good relationship between PAL activity and resistance to Botryosphaeria blight for the tested cultivars. This was probably because of variation in tree conditions among different cultivars and within each cultivar.

### **Project Evaluation**

Additional experiments need to be performed until any of the findings in this study can be adapted in the field. Experiments using varying nutrient amounts are considered difficult, but the experiments performed in this study can serve as a foundation for further experimentation. There were several difficulties encountered in this project. For instance, before initiating the fertilization experiments, leaching all the nutrients from the pots (or plastic sleeves used as pots) of the trees was almost impossible. A second problem was that this was the

first time we performed such experiments using various amounts of nutrients in potted pistachio trees, and in a way, we were experimenting with choosing and perfecting the methodologies. A third and most important problem was the loss of the majority of our freezer samples. This is the first study of this kind involving nutrients, pistachios, and diseases. The initial results indicate that indeed that these results may have some application in the field, but after the completion of more greenhouse and lath-house studies. However, it is generally believed that trials in the field, although they could be more difficult than those in the greenhouse, might generate results that could be easier adopted by growers.

### **Outreach Activities:**

11/8/2000 “Foliar and fruit diseases of pistachio (part I)”, Pistachio Production Course, UC Cooperative Extension, Visalia, CA; 130 participants: growers, PCAs, pistachio producers and packers, farm managers from abroad and researchers of foreign institutions.

11/14/2000 “Relationship between fertilization and pistachio diseases”, Eight Annual Fertilizer Research and Education Program Conference, Tulare, CA; 45 participants: growers, farm advisors, PCAs, fertilizer dealers, other UC and State researchers, USDA researchers.

01/16/2001 “Management of Botryosphaeria blight”, 2001 Pistachio Day, Visalia, CA; 200 participants: growers, PCAs, chemical company representatives, fertilizer dealers, USDA and DFA representatives.

02/06/2002 “Fertilization & Pistachio Disease”, 2002 California Plant and Soil Conference, California Chapter of American Society of Agronomy and California Plant Health Association, Fresno, CA; 50 participants: agronomists, farm advisors, PCAs, fertilizer dealers, UC and State researchers, USDA researchers, and nurserymen.

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**Table 1.** Effects of various nutrients on Botryosphaeria blight of pistachio caused by *Botryosphaeria dothidea* in a greenhouse experiment (1999).

Treatment	Infected leaves %	Leaf disease index
75% N	64.4 <sup>x</sup> a <sup>y</sup>	0.90 <sup>x</sup> ab
75% P	60.4 a	0.95 a
75% K	62.7 a	0.83 ab
100% N,P,K	58.8 a	0.87 ab
200% N	60.1 a	0.80 abc
200% P	57.3 a	0.71 bc
200% K	54.3 a	0.58 c

<sup>x</sup> Data in presented in columns are the average of two experiments because the two experiments were virtually the same

<sup>y</sup> Values with different letters are significantly different at  $P = 0.05$  level according to LSD test.

**Table 2.** Effects of various nutrients on Botryosphaeria blight of pistachio caused by *Botryosphaeria dothidea* in a greenhouse study in 2000.

Treatment	Disease index (DI)
<b>Experiment I</b>	
CK (100% N, P, K)	0.745 <sup>x</sup> ( $\pm 0.281$ ) <sup>y</sup> a <sup>z</sup>
50% N	0.910 ( $\pm 0.280$ ) a
200% N	0.527 ( $\pm 0.173$ ) a
50% P	0.885 ( $\pm 0.416$ ) a
200% P	0.598 ( $\pm 0.090$ ) a
50% K	0.730 ( $\pm 0.145$ ) a
200% K	0.522 ( $\pm 0.096$ ) a
0.1% CaCl <sub>2</sub>	0.568 ( $\pm 0.152$ ) a
0.2% CaCl <sub>2</sub>	0.527 ( $\pm 0.181$ ) a
<b>Experiment II</b>	
CK (100% N, P, K)	0.468 ( $\pm 0.114$ ) abc
50% N	0.488 ( $\pm 0.048$ ) ab
200% N	0.440 ( $\pm 0.100$ ) abcd
50% P	0.528 ( $\pm 0.100$ ) a
200% P	0.403 ( $\pm 0.056$ ) bcde
50% K	0.475 ( $\pm 0.093$ ) abc
200% K	0.340 ( $\pm 0.029$ ) de
0.1% Ca(NO <sub>3</sub> ) <sub>2</sub>	0.315 ( $\pm 0.055$ ) e
0.2% Ca(NO <sub>3</sub> ) <sub>2</sub>	0.358 ( $\pm 0.046$ ) de
0.4% Ca(NO <sub>3</sub> ) <sub>2</sub>	0.378 ( $\pm 0.057$ ) cde

<sup>x</sup> Data presented are the average of four replicated trees.

<sup>y</sup> Numbers in ( $\pm$ ) denote standard errors.

<sup>z</sup> Values in the column for each experiment followed by the same letter are not significantly different according to LSD of SAS test at  $P = 0.05$ .

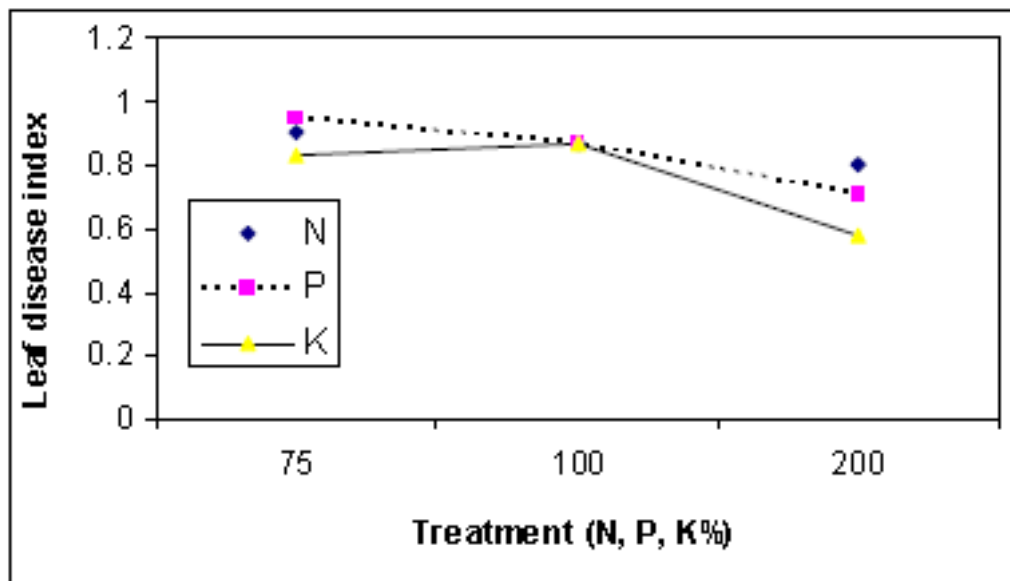
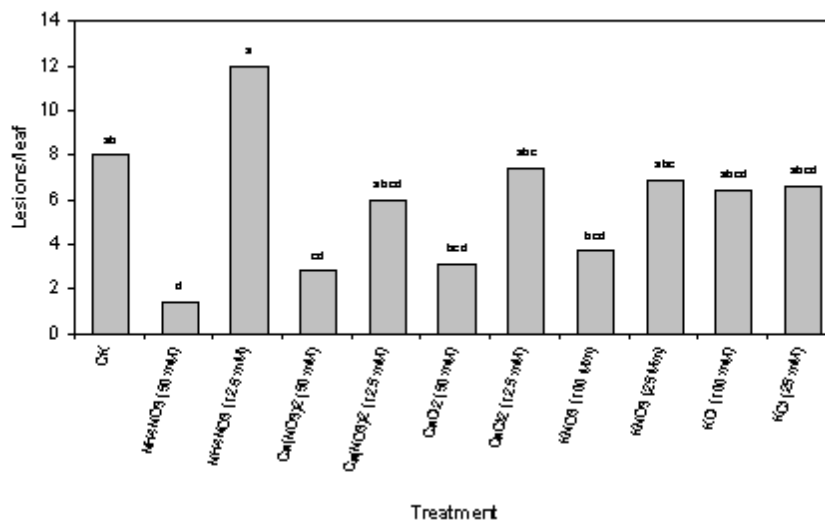
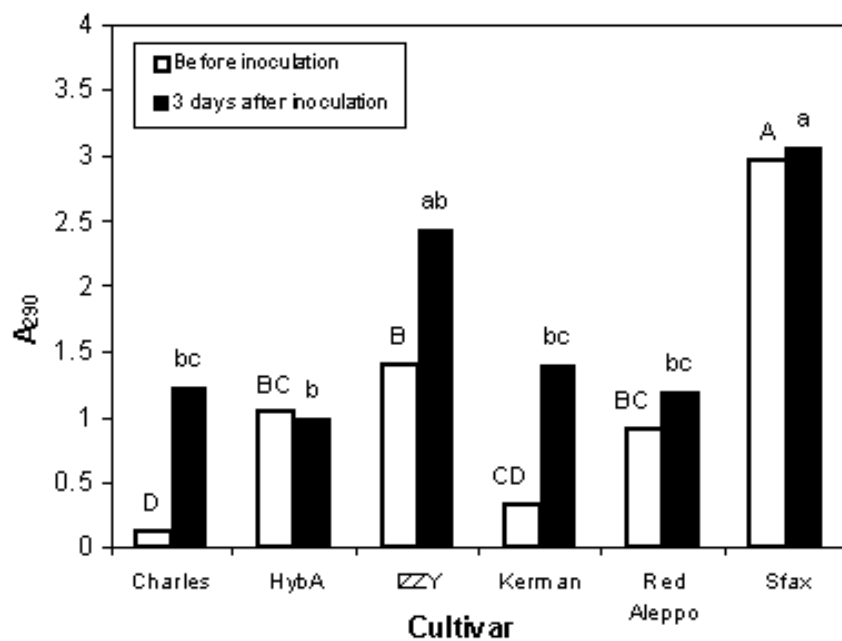


Figure 1. Effect of various fertilizers on *Botryosphaeria* blight of pistachio caused by *Botryosphaeria dothidea* (greenhouse experiment).



**Figure 2.** Effects of nutrition stress on *Alternaria* late blight of pistachio in greenhouse. Values with different letters are significantly different at  $P = 0.05$  level according to LSD test.



**Figure 3.** Comparisons in phenylalanine ammonia lyase (PAL) activity among 6 pistachio selections *Pistacia vera* cultivars before and after inoculation with various biotypes of *Botryosphaeria dothidea* in California.

### **Protocol for Overnight Freezing Incubation Technique (ONFIT)**

1. Disinfect cleaned plastic screen racks and crispers (5 per site) in 1:20 bleach (5.0% sodium hypochlorite: water) solution for five minutes. Remove from solution and drain with screen racks inside the crispers and lids in place. Allow covered crispers to sit until required. Label the crispers as desired. Have on hand at least 5 L of autoclaved tap water in sealed flasks.
2. Sort and count out 18 healthy-appearing leaflets per tree to be tested.
3. Place 9 leaflets in the plastic mesh-stretch bags, label as desired, and secure mesh bags with plastic clips.
4. Prepare bleach solution in large (20 L) plastic containers. Solution is prepared 1:10 with 0.5ml of Tween-20/liter of tap water. Typical preparation is 5 L of solution with 2.5 ml of Tween-20. Place the plastic container in the sink to minimize distribution of the bleach solution.
5. Prepare 1 L of 70% ethanol in a 2 L beaker.
6. In groups of two to four bags, place the samples in the ethanol solution for 10 seconds, shake off quickly and place in the bleach/Tween-20 solution for 4 minutes. Use a timer for consistency. Swirl the bags in the solution for 5 to 10 seconds during every minute the bags are in the solution.
7. At the end of the allotted time, remove the bags, shake off the excess liquid in the sink and quickly place the bags in the appropriately labeled crispers. Replace the lid.
8. Turn on the lights and air to the laminar-flow hood and disinfect the interior with 70% ETOH. Allow the hood to dissipate the alcohol fumes prior to starting work. Sterilize two large tweezers and place on a sterile surface to cool.
9. Move a crisper under the hood and remove the lid. Pour 200 ml of the sterile tap water into the bottom of the crisper. Under the hood, remove the samples from the bag by shaking out or use the tweezers to carefully remove samples stuck in the mesh of the bag.
10. Arrange 9 leaflets in rows of three in the crispers. Cover the crisper prior to removing from the hood.
11. Move all crispers to  $-16^{\circ}\text{C}$  freezer for about 15 hours (5:00 p.m. to 8:00 a.m.) overnight.

12. Remove crispers from freezer and place on a counter at 27-30°C (30°C is optimum).

13. Record and count disease lesions after 3, 7, and 10 days.

Note: A similar procedure can be used by using fruit samples collected from orchard trees.