AECOM-UC Davis collaboration: Identification and characterization of fungal pathogens infecting Opuntia in San Diego County

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Challenge

In 2021, *Opuntia littoralis* (Coastal prickly-pear) plants at a restoration nursery in San Diego County were observed with circular yellow, red, and gray dry lesions surrounding their spines. In 2022, further field surveys across 10 restoration sites in San Diego County showed affected plants with similar decay symptoms, at 5-25% prevalence. Cacti decay observed in restoration nurseries and in native habitats created concern about pathogen introductions into ecosystems that support coastal cactus wrens (a species of concern in CA).

Project objectives and results

1. **Objective 1:** Determine pathogenicity of *Alternaria alternata* isolates in live Opuntia plants and investigate what environmental factors impact disease virulence

1.1. Pathogenicity trials in the greenhouse

Methods.

Plant maintenance and experimental replicate conditions. At the Orchard Park greenhouses in Davis, California, two identical pathogenicity trials were conducted—Trial 1 with 48 *Opuntia littoralis* plants from the San Pasquale Battlefield State Park ("Battlefield") location, and Trial 2 with 48 *Opuntia littoralis* plants from the Bernardo Mountain-Lake Hodges ("Bernardo Mountain") location. AECOM field ecologists took single-cladode cuttings from Opuntia littoralis with no visible symptoms at these locations and mailed them to the Del Castillo lab in Feb 23, 2024 (Battlefield) and May 26, 2024 (Bernardo Mountain). The cladodes were left to callus on the bench for seven to twelve days, planted in 1 gallon pots containing UC Agronomy mix (equal parts sand, redwood sawdust, sphagnum peat moss, and pumice rock mixed with 1% dolomite lime), then maintained with 100mL of water every five days for five months (Battlefield) or 10 months (Bernardo Mountain). Trial 1 was conducted from October 27, 2023 to February 9, 2024, and Trial 2 was conducted from March 15, 2024 to June 28, 2024. Average recorded temperature inside the greenhouse was 27±3.7°C during Trial 1 and 30±6.59°C during Trial 2.

Experimental design. To evaluate the effect of environmental factors that trigger disease severity, humidity and irrigation treatments were evaluated. Humidity levels consisted of high humidity (70% average) or low humidity (36% average). Irrigation treatment consisted

of sufficient water (100mL every 5 days) or low water (100mL every 10 days). The experiment was arranged as a randomized complete block split plot design, with two blocks. The main plot consisted of the humidity treatment, which were randomly distributed in each of the two blocks. Irrigation and pathogen treatment were in the subplots, consisting of three inoculated and three non-inoculated plant randomly distributed within each main plot for a total of six plants per pathogen, irrigation, and humidity treatment combination per block (Figure 1).

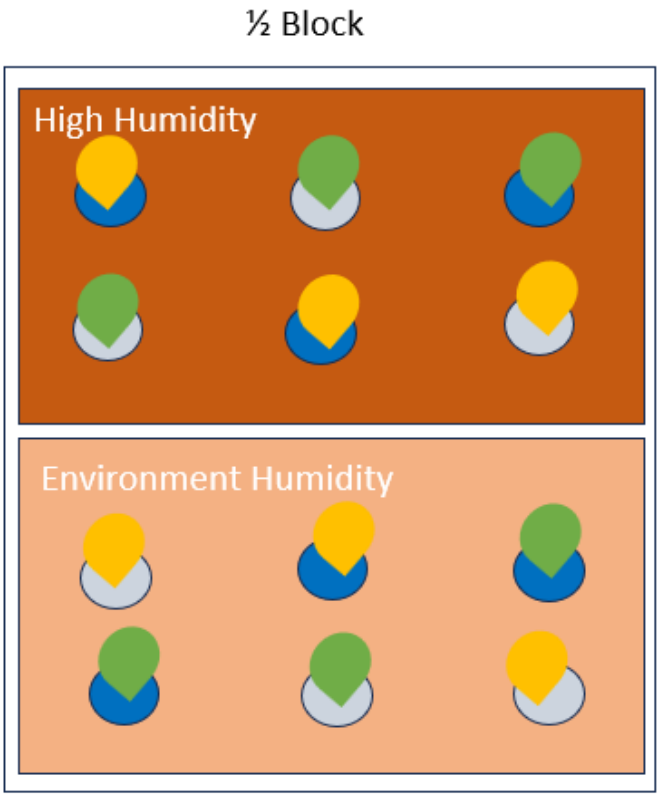

Figure 1. Effect of humidity and irrigation on Alternaria leaf spot experimental design. The experiment was arranged in a randomized complete block split plot design. Humidity treatment is the main plot. Subplots consist of the irrigation treatment (light blue circles indicate low water; dark blue circles indicate sufficient water) and the pathogen treatment (yellow ovals indicate inoculated plants; green ovals indicate non-inoculated plants).

Treatment application. *Alternaria alternata* isolate OP029, the most virulent isolate from our lab pathogenicity trials, conducted in 2023, was used to inoculate plants for the greenhouse experiment. A 10^5 spores/ml spore suspension was created by scraping 14-day-old OP029 cultures into a sterile 0.5% KCl solution. Plants were inoculated in the greenhouse by being turned on their sides so that the cladode was flat and facing the roof, then being wounded with a sterile probe in three areas, two close to each of the lateral margins in the widest portion of the cladode, and one at the center of the cladode, 5cm from the bottom. Each wound area consisted of four wounds in an approximately 2cm square area. 25ul of the spore suspension was pipetted onto each wound. Non-inoculated

plants were wounded in the same way but a 0.1% water agar solution was pipetted onto them instead. 24 hours later, the pots were moved upright.

Plants with the low humidity treatment were kept on the bench in the greenhouse, where the average humidity during the two experiments was 36%. Plants with the high humidity treatment were kept in humid chambers in groups of six, where the average humidity during the two experiments was 70%. Plants with the sufficient water treatment were given 100mL of water every 5 days through drip lines. Plants with the low water treatment were given 100mL of water every 10 days through hand watering.

Cladode lesions and pathogen reisolations. Lesion size was measured in millimeters every three weeks for a total of fifteen weeks per experiment using calipers. The lesion size of each wound was measured. Lesions were defined as yellow, gray, black, or red discoloration originating from a wound. This definition is based on previously observed lesions caused by *Alternaria alternata* from diseased plants from the area. Upon experiment completion, symptomatic tissue was processed on a fungal selective medium (1/10 PDA + tetracycline). Recovered colonies were purified and fungal DNA was extracted. PCR products of the ATPase region were submitted for sanger sequencing and were analyzed using BLAST.

Statistical analysis was performed in R using one- and two-way analysis of variance tests and Tukey's HSD tests.

Results

Trial 1 (Battlefield)

Within one week post-inoculation, inoculated plants were observed with yellow, gray, black, or red circular lesions (Figure 2). Non-inoculated plants never developed lesions whereas the lesions on inoculated plants continued growing throughout the 15 weeks (Figure 3). Pathogen treatment had a significant effect on lesion size ($P < 0.001$); plants inoculated with *Alternaria alternata* isolate OP029 had significantly larger lesions (5.03 mm on average) compared to non-inoculated plants.

Within inoculated plants, irrigation and humidity treatments were not significant ($P = 0.14$ and $P = 0.80$, respectively). However, their interaction was significant ($P < 0.05$). Inoculated plants in high humidity developed larger lesions (6.72 mm on average) if they had sufficient water compared to plants with low water (3.54 mm on average) (Figure 4).

Alternaria alternata was recovered from inoculated cladodes submitted for molecular identification, and not recovered from non-inoculated controls.

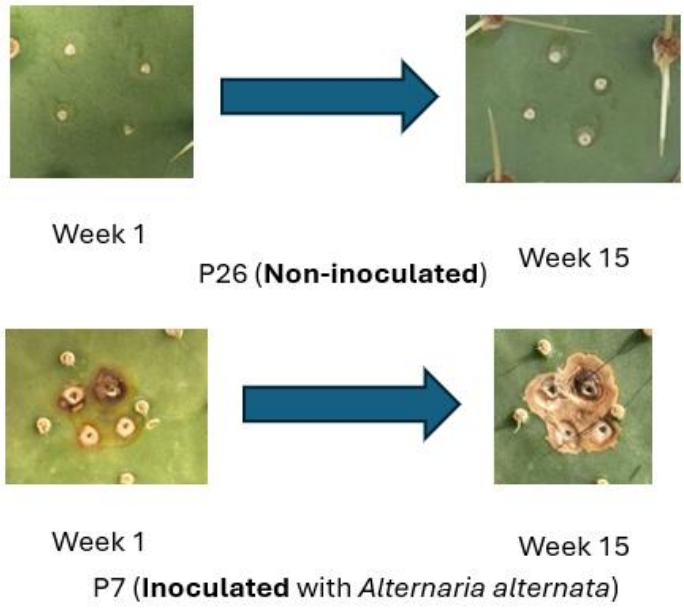

Figure 2. Lesions from two plants from week 1 and week 15 of Trial 1. P26 was non-inoculated and had sufficient water and low humidity; it did not have lesion growth. P7 was inoculated and had low water and low humidity; it had lesion growth during the 15 week trial. The lesions on P7 are reddish yellow.

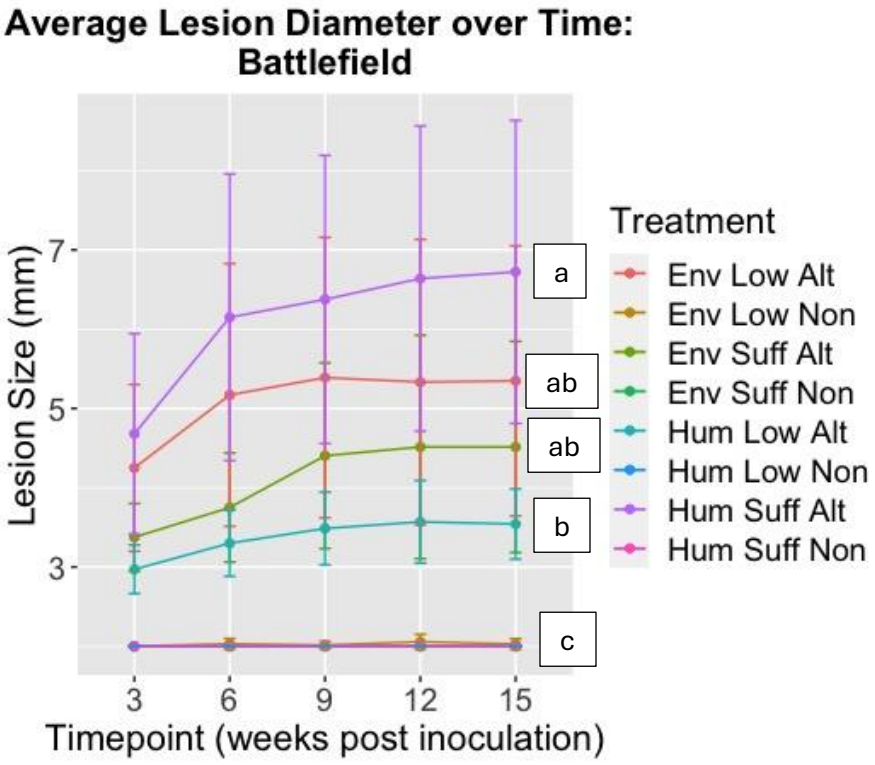

Figure 3. Graph showing lesion size in millimeters over time for Trial 1. Each line is a different treatment; the non-inoculated treatments didn't develop lesions whereas the inoculated treatments developed lesions that grew over the course of the experiment.

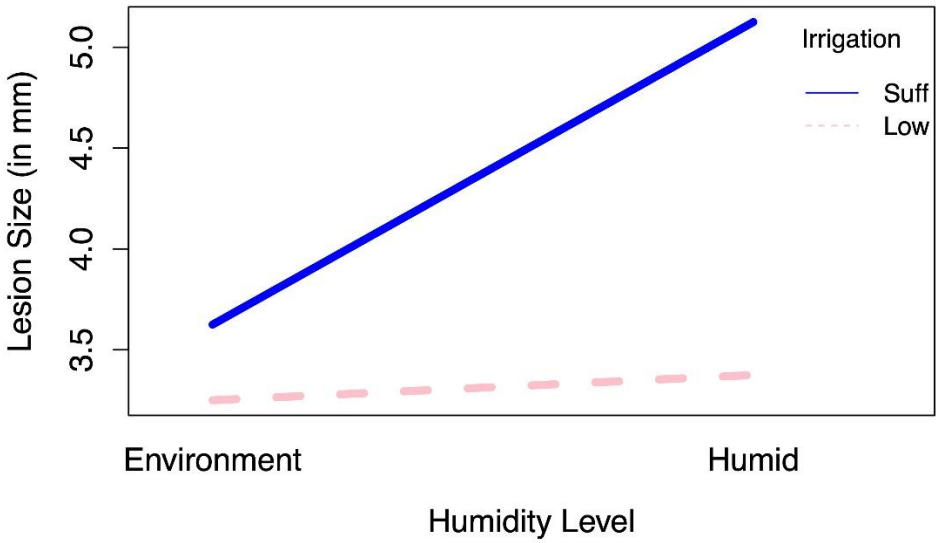

Figure 4 . Interaction plot with the humidity treatment on the x axis, the irrigation treatment as the two lines, and the dependent variable, lesion size in millimeters, on the y axis. An interaction effect between the two treatments is visible because the lines are not parallel.

Trial 2 (Bernardo Mountain)

As in Trial 1, inoculated plants developed yellow, gray, black, or red circular lesions that were noticeable within a week of inoculation, grew over time, and originated from the inoculation site. Non-inoculated plants did not develop lesions (Figure 5). Pathogen treatment had a significant effect on lesion size ($P < 0.001$); plants inoculated with *Alternaria alternata* isolate OP029 had significantly larger lesions (4.73 mm on average) compared to non-inoculated plants, regardless of their environmental conditions (Figure 6).

Within inoculated plants, neither irrigation level nor the interaction between irrigation and humidity had a significant effect on lesion size ($P = 0.39$ and $P = 0.19$, respectively). However, humidity did have a significant effect on lesion size ($P < 0.001$); inoculated plants in high humidity developed larger lesions (5.14 mm on average) than inoculated plants in low humidity (4.39 mm on average) (Figure 7).

Alternaria alternata was recovered from inoculated cladodes submitted for molecular identification. No symptoms were observed on non-inoculated controls throughout the entire experiment.

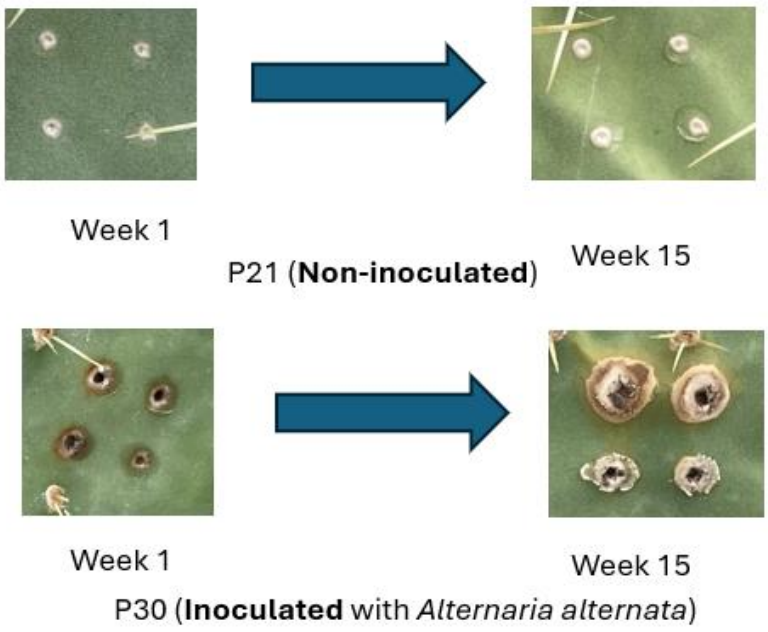

Figure 5. Lesions from two plants from week 1 and week 15 of Trial 2. P21 was non-inoculated and had low water and low humidity; it did not have lesion growth. P30 was inoculated and had sufficient water and low humidity; it had lesion growth across the 15 week trial. The lesions on P30 are reddish gray.

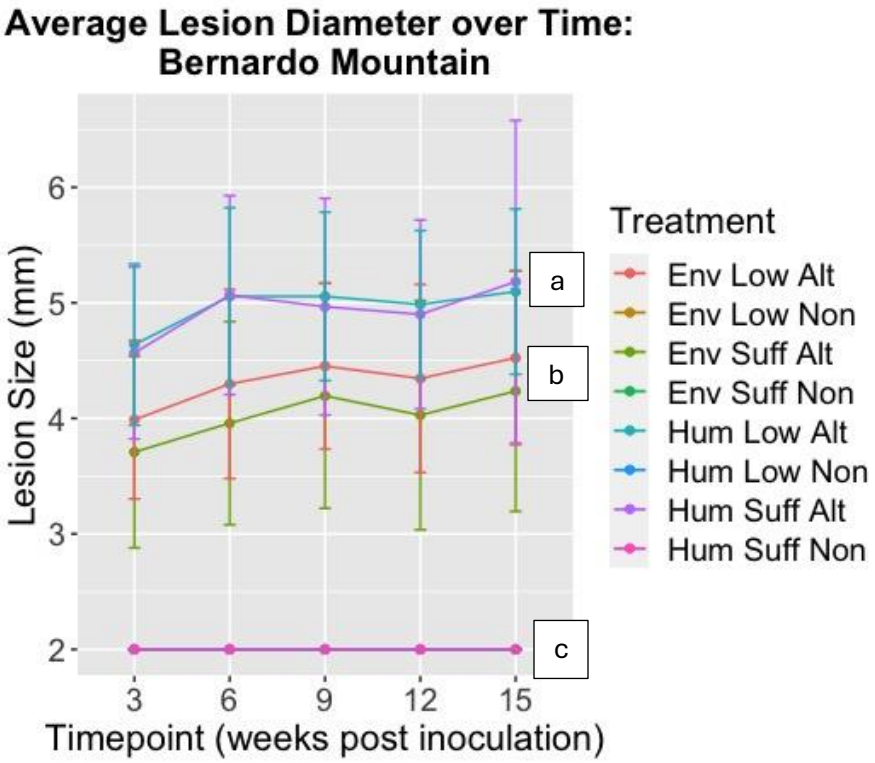

Figure 6. Graph showing lesion size in mm over time for Trial 1. Each line is a different treatment; the non-inoculated treatments didn't develop lesions whereas the inoculated treatments developed lesions that grew over the course of the experiment.

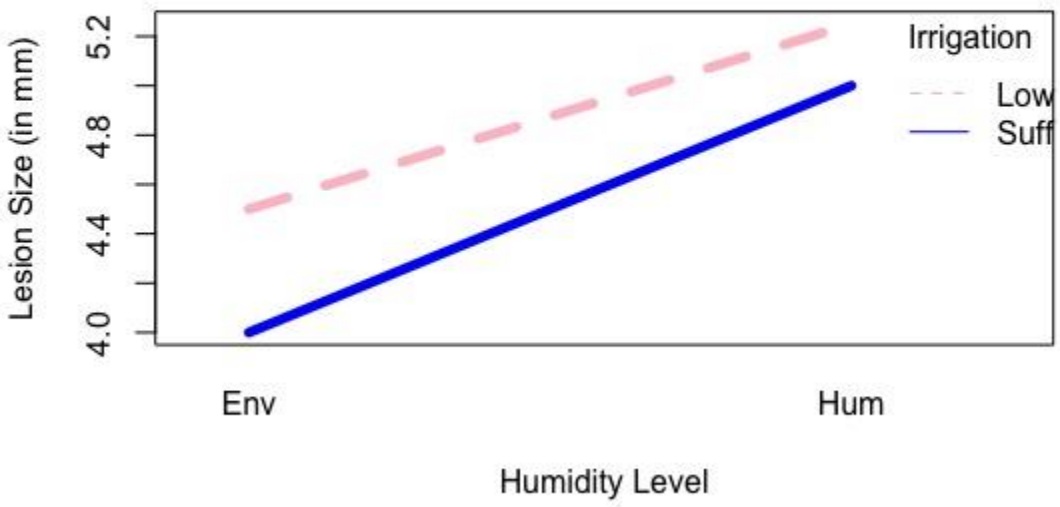

Figure 7. Interaction plot with the humidity treatment on the x axis, the irrigation treatment as the two lines, and the dependent variable, lesion size in mm, on the y axis. The lines are almost parallel, indicating that there is no interaction effect between the two treatments.

Conclusions and recommendations

In both trials conducted in our greenhouse, we found that live, previously healthy *Opuntia littoralis* plants that were inoculated with *Alternaria alternata* developed significantly larger lesions than non-inoculated plants. These lesions looked similar to lesions we observed on cladodes with naturally occurring *A. alternata* infections from these sites in 2021 and 2022. Because the lesions observed were similar and we successfully recovered *A. alternata* from inoculated and symptomatic plants, we completed the last part of Koch's postulates. Therefore, *Alternaria alternata* can cause disease in previously healthy *Opuntia littoralis* plants. As inoculated cladodes from both the Battlefield and Bernardo Mountains locations developed lesions, *Opuntia littoralis* does not have a natural resistance based on location.

The environmental treatments of irrigation level and humidity had different effects on lesion size in the two different trials. Experimental results show that humidity has an effect in increasing lesion size caused by *A. alternata*. In the first trial, when the plants were in humid conditions and had sufficient water, they developed larger lesions than plants that were in humid conditions but had low water. In the second trial, plants that were in humid conditions developed larger lesions, regardless of their irrigation levels. Based on this information from both trials, it is recommended that nurseries maintain low humidity, and

prevent over-irrigation to manage *A. alternaria* infections. At restoration sites, if plants are infected, symptoms may be exacerbated during the rainy season or at locations with close proximity to the ocean.