Understanding Black Heart and Botrytis Gray Mold of Pomegranate and Management of These Diseases

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Introduction and Purpose:

General on management of black heart disease. Over the last decade-plus, black heart of pomegranates has become a major concern for growers in the pomegranate industry. Although the fruit looks “perfect” on the outside, when cut open, sections or the whole interior of the fruit may be covered with a dark grey to black mass of mold and decay. Advanced infection may result in fruit that is less dense with a mottled and puffy rind that often has darkened coloration. As a result, there is some potential to sort and screen out severely infected pomegranates. However, many infections do not display any external cues about the extent of infection within. In our experience, the typical extent of infection is over 50% of the volume, but may also be limited to a discolored lesion the size of a dime. Infection rates in the field have been estimated as high as 10% for cv. ‘Wonderful’ harvested from certain fields, and up to 20% for other cultivars in Cyprus (Kahramanoglu, 2014). For pomegranate growers, any time that a consumer cuts open a pomegranate with black-heart there is potential for harm to product appeal and any costs associated with reduction of infection rates may pay for themselves in consumer confidence.

When studies of black heart were initiated in the Michailides’ laboratory in 2006, it was not clear whether or not a single pathogen was involved in causing black heart of pomegranate. Due to the lack of any scientific studies to consult, growers were very confused about this decay and referred to *Alternaria, Aspergillus, or Botrytis* as the cause of black heart. Parallels could be drawn from moldy-core of apple. *Alternaria* spp. were known to infect the apple core as a result of moving through the open calyx in certain cultivars of apple (Ellis, 1983). After multiple isolations from pomegranates showing black heart symptoms and confirming Koch’s postulates, it became apparent that mainly *Alternaria alternata* and occasionally other *Alternaria* species were the main causal agents of black heart disease of pomegranate. Additionally, the patterns and frequency of the disease and the mode of infection by the pathogen were without documentation. Based on counts made with fruit from the experimental block of Wonderful pomegranates at the University of California Kearney Agricultural Research and Extension Center (KARE) and from large commercial blocks at Paramount Farming Company, we have found that there is considerable variation in the rates of infection across years and between different locations. In fact, low infection rates at the UC KARE Center (less than 1 percent) pushed us towards cooperation with the industry for larger field trials in 2012 and 2013. The industry has reported infection rates for given blocks in excess of 10%. A block that yielded a 10% rate one year may only have an infection rate of 2% the following year. This variation may largely be the result of environmental conditions that promote greater susceptibility and influence the rates of infection from year to year and location to location. As for varieties,
Karhamanoglu et al (2014) reported infection rates of 20.3, 14.9, and 9.8% for Acco, Herskovitz and Wonderful cultivars, respectively, for pomegranates cultivated in field trials in Cyprus. There is another cultivar, Kara Gul, grown at the Wolfskill Experimental Orchards, of the University of California in Winters, CA, which appears to have a greater infection rate than cv. Wonderful (E. Wilkins, personal communication). With the assistance of Paramount Farming Company, we are in the process of establishing a block of cv. Kara Gul for future experiments at UC KARE.

The two most important objectives in our pomegranate studies were to determine the path of infection and whether any chemical treatments can reduce the number of infections in the field. The flowering stage has been suggested as being the most likely window for infection (Ezra, 2013) and this inference has been reinforced by studies in 2006 which yielded the greatest rate of infection for blossoms inoculated at full bloom (Michailides, 2008). The thick, leathery rind seems like a resistant barrier against potential pathogens and although there is cracking of the fruit in the late season and potential for puncture wounds from thorns, the nature of infections leading to black heart did not seem consistent with entry by these means. Infection appears to initiate from within the pomegranate. Therefore, it was hypothesized that conidia of *Alternaria* may travel in conjunction with the pollen grains during pollination. Under the microscope, it appears conidia of *Alternaria alternata* are roughly 1/4th the size of the cv. Wonderful pollen grains and would have the potential to migrate to the interior of the fruit. Over the course of examining multitudes of black heart infected pomegranates over multiple years and from distinct locations, our hypothesis has evolved regarding the pathway of infection for black heart. Ezra et al (2013) reported at the 3rd International Symposium on Pomegranates that “the fungus causing the disease (*Alternaria* spp.) is penetrating the flower causing disease later in fruit development”. From Ezra’s studies in Israel, he found that over 90% of the pomegranate blossoms were inhabited by *Alternaria* spp. The author suggests movement by fungi though the tunnel into the interior of the fruit: *Alternaria* extended beyond the tunnel to the “locules” in 8% of the fruit examined and 5% of the fruit in the orchard had black heart symptoms. Our studies in 2013 had remarkably similar observations that support this hypothesis.

Targeting sprays at full bloom seems like a promising approach. However, a significant challenge is posed by the indeterminate bloom pattern of pomegranates. In California’s San Joaquin Valley blooms may initiate and set fruit in early April; but fruit set will continue throughout the summer as blossoms are continuously born on terminal shoots. Peak bloom in one block may fall a week later than in an adjacent block and rows within the same block may not be in synchrony. When a pomegranate orchard is flush with red blossoms, yet not considered to be in full bloom, it seems that a paradox is at hand. This has to do with the nature of the blossoms: pomegranate blossoms have dioecious characteristics in that within the same tree there are perfect flowers possessing a pistil and anthers (hermaphrodites) and there are male flowers with anthers and degenerate or nonexistent pistils. Hermaphrodite flowers are characterized by a longer bell shape hypanthium than the more conical male inflorescence. It is the hermaphroditic blossoms that are pollinated, swell towards the stem end, and develop into fruit while the male flowers abscise shortly after bloom and fall to the ground. However, the proportions of male and hermaphrodite blossoms present throughout the prolonged bloom period are not constant. There appears to be a pattern in which the proportion of hermaphrodite blossoms increases towards a peak and this stage is considered full bloom. Levin (2006) reports
that the proportion of male to ‘bisexual’ blossoms may be related to availability of assimilates and freeze damage; when there are insufficient resources for ovary development, development is steered towards male structures. Something to consider for further investigations is whether or not the proportion of male blossoms could be an indicator of susceptibility to disease. Perhaps the same shortage of resources affecting determination of blossoms affects the ability of tissues within pollinated blossoms to withstand infection. A study in Greece was conducted to examine how the ratio of male to hermaphrodite blossoms shifts over the course of a growing season (A. Manganaris, 2011). They found that at the beginning of bloom, the predominance of blossoms were male and within 2-3 weeks the hermaphroditic blossoms began to steadily increase as male inflorescence steadily declined, then picked back up. There appears to be equal proportions of male and hermaphrodite blossoms at what appears to be “full bloom”. It does not appear that data collection continued after this point, but in our observations, the production of hermaphrodite blossoms appears to drop off after a critical number of fruit have been set, while the proportion of male blossoms that emerge throughout the growing season remains relatively high. Consistent with the theory regarding assimilates; Levin (2006) suggests heavier crop set reduces the rate of hermaphroditic blossoms in successive blooms and likely has an effect on the nature of the bloom characteristics for the following season.

Recognition of full bloom is not without challenges but may be critical to successful management of black heart. After all, full bloom is when the greatest value of the crop is set and when the greatest losses due to fungal decay can be initiated. Given the importance of building and maintaining consumer confidence in this expanding market, it is imperative for the industry to find a cost effective method for reducing the frequency which infected pomegranates reach the consumer. From the assumptions about the timing of disease entry and the timing of full bloom, it seems possible that well timed chemical applications may inhibit the ability of *Alternaria* to inhabit the blossoms and may restrict the travel of infections though the pollen tube.

The objectives of our study on black heart were to investigate i) the infection pathway by the pathogen; ii) the effect of inoculation timing and confirm previous findings; and iii) the effects of fungicides sprays during bloom against black heart disease.

**Management of gray mold infection.** Another focus of our study in 2013 was on a disease of pomegranate that is better understood than black heart and more readily found in the literature. Gray mold caused by *Botrytis cinerea* is considered the most significant of post-harvest disease affecting pomegranates. Before the existence of registered fungicides, gray mold was responsible for approximately 30% of post-harvest losses (Tedford, 2005). A 2001 emergency exemption registration resulted in the availability of Scholar (active ingredient fludioxonil) for use as a post-harvest dip. Tedford reported an 80% decrease in crop losses as a result of the fungicide treatment. Scholar is still in use in packing houses today, but in spite of its effectiveness, latent infections from calyces that were initiated pre-harvest lead to gray mold in post-harvest and approximately 5% losses, regardless of fungicide treatment. Research by Palou et al (2013) has scrutinized the nature of the latent infections that inhabit pomegranates of the Mollar de Elche cultivar in Spain. In their findings, *Botrytis cinerea* was the most commonly isolated pathogen from latent infections. Similar to pomegranates, kiwifruit are also susceptible to gray mold in cold storage as a result of latent infections that inhabit the calyx and receptacles. Research by Michailides et al (1996) monitored the rates of latent infection for kiwifruit in 9
vineyards and correlated these results with the incidence of gray mold after 3 and 5 months. By sampling the kiwifruit at intervals after fruit set, they found a high correlation for latent infection at 4 months after fruit set with gray mold damage in cold storage. The authors suggest that this information may assist packinghouse managers in determining which fruit to sell first and which can be stored longer without heavy losses due to gray mold. The methodology from that experiment has been adapted for current studies in pomegranates where sections of the calyces are sterilized and plated on growth media. Additionally, in the same paper, experiments were conducted to determine if pre-harvest sprays had an effect on gray mold incidence in cold storage. For the vineyard with the highest incidence, the sprays significantly reduced gray mold for applications one week before harvest (Michailides, 1996).

The objectives of our study on gray mold were to i) investigate the correlation, if any, between incidence of latent infections pre-harvest and infection arising in cold storage; and ii) test the efficacy of fungicides sprayed at pre-harvest intervals for control of gray mold on pomegranates.

**Fungicide screening for other potential pomegranate postharvest pathogens.** Over the course of examining pomegranates that are infected by gray mold during post-harvest, the presence of other fungal pathogens with the potential to cause economic losses has been documented in our lab. The two fungi that appear to have pathogenic potential, as confirmed by Koch’s postulates, are *Pilidiella granati* and a putative *Monilia* species whose identity has yet to be verified at the molecular level. Our objective was to screen in vitro isolates of these fungi against fungicides used in our field trial during bloom and the registered fungicide Scholar® that is used by the industry to reduce gray mold damage postharvest.

**Materials & Methods:**

**Blossom inoculation.** Towards late April of 2013, our Paramount Farming cooperators began identifying potential blocks for a fungicide trial at Dudley Ridge in Kern County. It was essential that we set up the experiment in blocks that were expected to reach peak bloom on the day we were to inoculate the blossoms. Ranch 2610, Block 15-01 was the first to meet the criteria and was selected for week one inoculations. This block was also selected for the first week of inoculations in 2012. Ranch 2590, Block 05-03 was selected for the second week. Six rows and 7 trees from the row ends were utilized as buffers from the roads that divide the blocks. Five rows, one for each set of replicates, were set aside for the experiment. For each row, the number of trees used was dependent on the density of unfertilized female blossoms at the optimum stage. The density of female blossoms can be highly variable from row to row and tree to tree; some trees set bloom with predominantly male flowers, which dehisce and fall to the ground shortly after bloom. Fifty female blossoms were targeted for each of the 5 treatments within a replicated row. The first week of inoculations began on May 1, 2013. Shoots with female blossoms (usually one per shoot, but occasionally two) at the optimum stage of development were selected and flagged with the appropriate color/pattern within the randomized block design. The criteria for optimum bloom phase were based on the extent of the calyx aperture; when the tips of the sepals no longer point inward and the petals are just beginning to emerge behind the sepals. Given the warm temperatures this time of year in the south Central Valley, these optimal stage blossoms are highly likely to be in full bloom within less than 24 hours; and this is the stage at which inoculation can lead to the highest infection rates. Previous
research by Michailides et al (2008) concluded that the optimum stage for artificial infection is
when petals are open and the anthers have yet to dry. Targeted blossoms (1250 total) were
flagged and tagged according to treatment by a Paramount Farming crew (including our staff, 7-
8 individuals on a given day) with individual blossoms tagged with a number for identification
purposes. The 5 treatments consisted of Pristine® (pyraclostrobin+boscalid), Luna® Experience
(tebuconazole+fluopyram), Polyoxin-D, inoculated control, and non-inoculated background
control. After the departure of the field crew, each chemical treatment was sprayed with a
handgun spray nozzle from a 4-tank sprayer custom designed by Rear’s Manufacturing©. All
chemicals were applied at maximum recommended field rates for other tree crops. Pristine®
was applied at 14.5 oz/A or 30.9 g/30 gal; Luna® Experience was applied at 6 oz/A or 13 mL/30
gal; and Polyoxin-D was applied at 6.2 oz/A or 13.2 g/30 gal. Whole trees were sprayed, but
shoots with blossoms were targeted to ensure coverage. The following day, the field crew
returned to assist with inoculating and bagging the flagged shoots. A spore suspension
consisting of 4 different isolates (1I43, 1J02, 3H26, and 5B81) of Alternaria alternata obtained
from naturally infected pomegranates was prepared at a 10⁴ conidia/ml density. Each blossom
was sprayed twice with a hand held mist sprayer and a third spray was directed inside a plastic
bag which was used to envelope the shoot and create a humid environment. A white paper bag
was secured around the plastic bag with a plastic tie to reflect as much solar energy as possible
although temperatures inside the bag may still exceed 100° F. The bags remained on the shoots
overnight and were removed the following morning. Procedures were repeated the following
week starting on May 8th in the other block. After standard cultivation practices by Paramount
Farms, the pomegranates were harvested on September 19th and 26th for the week one and two
blocks, respectively.

An additional experiment was added to the 2013 field trial at Ranch 2610, Block 15-01. In an
effort to corroborate earlier findings about the optimum window for initiating infection, an
additional 300 blossoms at the same developmental stage were flagged and tagged on the same
date as done with the blossoms for the week one inoculations. One hundred blossoms were
assigned ‘+1 Week’, another 100 were assigned ‘+2 Weeks’, and the final 100 given ‘+3
Weeks’. The inoculated control from the main experiment served as the ‘0 Week’ category. For
each successive week, the blossoms were inoculated with the same spore suspension (made fresh
each time) and bagged overnight as they were at the onset. The blossoms were fruitlet size by
the ‘+3 Week’ date. These pomegranates were harvested on the same date as the fruit from the
main experiment.

Pre-harvest sampling for latent Botrytis infections. Three separate blocks at Paramount’s
Dudley Ridge were selected for sampling based on historic rates of post-harvest gray mold
caused by Botrytis cinerea. Ranch 2320, Block 4-2, is located next to an almond block on the
west, open field and pistachio on the north, open field and equipment yard on the east, and
pomegranates to the south. Ranch 2320, Block 9-2 is surrounded by pomegranates. Ranch
2330, Block 10-A, is at a major traffic junction within the ranch, with a canal to the south and an
almond block to the southwest. Pre-harvest sampling was conducted at each block on August
21st, September 4th, 12th, 19th, and 27th. A final sampling was taken on the day of harvest,
October 3rd. Thirty pomegranates were randomly sampled within a localized area of each block.
These fruit were immediately transported to a 45°F cold storage room at the Kearney field
station and processed as soon as possible. The calyx of each of the 30 pomegranates from each
sampling was dissected for surface sterilization, plating, and incubation. With a scalpel, the triangular sections of the sepals of each fruit were sliced at the line where the sections merge so there were 4-7 individual pieces per calyx, and the remaining ring that constitutes the rest of the calyx was cut out and set aside. The ring section of the calyx was cut into 5-7 segments, each with filaments and anthers attached. The style and stigma was removed just below the top of the stylodium. These sections were then surface sterilized in 0.6% sodium hypochlorite with Tween for 1 min, allowed to dry in a laminar flow hood, and then plated on acidified PDA under the hood. Sections were plated so that a central piece was surrounded by 4-6 pieces with ample room for growth of fungi. For example, the style/stigma was plated in the center surrounded by the sepals. Plates were placed in a refrigerator whose temperature oscillated between 6-10 °C for 6 days. Evidence of potential Botrytis growth was recorded and the plates were set aside to incubate at room temperature (22-24 °C) for three days additional days. Fungi were identified and recorded when possible, some fungi required longer incubation for development of characteristic structures.

Harvest and cold storage of PFC pomegranates. Harvest of the sampling area within the three blocks occurred on 3 separate days and was facilitated by the Paramount field crew. Ranch 2330, B 10-A was harvested on October 3rd and packed on the 7th. The 2320 ranches were harvested on the 8th and 9th and packed on the same day as harvest. Parameters for fruit size (less than 100 mm diameter, and marketable size) and optimum appearance (free of cracks, wounds and sun-scald) were set. Over 600 fruit per block were loaded in bin and transported to Kearney Agricultural Research & Extension Center where they were immediately placed into cold storage. Boxes provided by PFC were lined with #11 trays and packed 2 layers deep with the stem end down. Twenty-three boxes for each of the three blocks were packed with 22 fruit each, labeled accordingly, and stack on shelves within a small cold storage room (21.4 m³) at 7.2°C and with 85% RH. Other than periodic inspection for development of gray mold, the pomegranates were left alone for 3 months until the first inspection in January.

Assessment of pomegranates following cold storage. Beginning on January 9th, 2014, boxes were removed from cold storage and each individual fruit examined for evidence of infection. Pomegranates showing infection by gray mold were removed, including fruit that were infected by contact with a primary source (Botrytis nesting effect). Fruit with discoloration in the crown or stem were incubated to verify the cause of infection. Boxes were scored separately for the total number of infections in the top layer and the bottom layer and returned to cold storage. Recovered isolates were preserved on APDA. An additional assessment was made for the pomegranates which did not develop Botrytis infections at the conclusion of the fifth month in cold storage. As was done for pre-harvest sampling, sections of the calyx and pistil were plated to determine presence of latent infection by B. cinerea.

Pre-harvest sprays at KARE to control postharvest gray mold. Thirty cv. ‘Wonderful’ pomegranate trees were incorporated in a pre-harvest spray trial at the Kearney agricultural field station. Whole trees were sprayed using the same Rear’s 4-tank sprayer with a hand gun nozzle that was used for the black heart field trial. Within a randomized block design, 5 trees were left as an unsprayed control, 5 for each of the four Pristine® treatments, and 5 for the Polyoxin-D treatment. On October 11th, ten trees received the first spray of Pristine two weeks ahead of harvest. The following week, on October 18th, one set of these trees received an additional
spray. A different set of five trees received treatment with Pristine one week ahead of harvest. Polyoxin-D was also applied to five trees on this date. On the day before harvest, October 24th, five trees were sprayed with Pristine.

**Post-harvest cold storage of KARE pomegranates.** Fruit that was harvested was to be without injury or cracking although concessions had to be made to fill boxes. The plan was to harvest 22 fruit per tree. Because some trees had minimal fruit or the fruit did not meet criteria, extra fruit from other reps were used to fill these boxes. We attempted to pack fruit that were free of cracks, but this could not be avoided as the late harvest and warm weather induced some surface cracking. Fruit were placed in cold storage immediately after harvest at 7.2°C and 85% RH. These pomegranates were assessed at the same 3 and 5 month intervals as the pomegranates from the Paramount blocks.

**Fungicide screening against *Pilidilla granati* and *Monilia* sp.** Several isolates from our -80°C glycerol collection that were originally obtained from infected pomegranates were used in the screening process. A few isolates were cultured directly from plates that were from preserved isolates collected during post-harvest observations and originated from Dudley Ridge or the Kearney field station. From the grams per liter concentration cited on the label of each product, calculations were made to determine the amount of product to be added to 0.5 L of potato dextrose agar (PDA) (Microtech Scientific, Orange, CA) in order to obtain a final concentrations of 10 µg/L of the total active ingredients for Luna® Experience, PhD®, Scholar®, Switch® 62.5, and Merivon®. Luna and PhD were tested at an earlier date than the other three products. After autoclaving and cooling the PDA, 1 ml from a calculated stock solution was added to the media and then poured into 9 mm Petri plates. When cooled and dried, 4 mm plugs taken from freshly cultured plates from each isolate were placed in the center of the amended and unamended (control) media. Four plates per isolate, per treatment were used. For the Luna and PhD testing, Pilidiella granati isolates were incubated at 30°C and Monilia isolates at room temperature (approximately 22°C) for 66 hours. Plates amended with Scholar, Switch and Merivon were all incubated at room temperature (22-24°C) for 96 hours. Radial growth was determined from half the average of the perpendicular diameters minus the 4 mm plug initially used to inoculate the plates.

**Results and Discussion:**

**Blossom inoculations.** In 2012 and 2013, we observed a wide range of background black heart infection rates. The background rates are indicative of the natural infection rates occurring at Paramount’s Dudley Ridge, however, there is a high degree of variability in black heart infection from block to block and week to week. In 2012, we observed a 10.7% background infection rate at Ranch 2610, Block 15-1 for blossoms tagged at the optimum stage on May 14th. Within the same block, for blossoms tagged on May 2nd 2013, the background infection rate was only 1.2%. Blossoms tagged one week later yielded an infection rate of 8.8%. It is possible that temperature and humidity play a role in the initiation of infections at bloom. That is not within the scope of these experiments, although CIMIS data was noted for all dates of the field trials. During the 2013 trial, CIMIS data from the Kettleman City station gives a max temperature of
93.4 °F with average relative humidity of 18% for the day of week one inoculations (May 2nd). The following week, the maximum was 83.4°F with RH of 44% (May 9th). Perhaps the decrease in temperature with increase RH has an influence on the incidence of infection during the opportune window of infection. By comparison, the weather records from the corresponding dates of the 2012 trial (16 and 22, May 2012) show a maximum of 92.8 °F with RH of 47% and 88.3 °F with 42% RH, respectively.

The data confirms that the inoculation method was very effective at increasing the infection rate of the pomegranates. By bagging the blossoms immediately after inoculation, the creation of a warm humid environment favors the germination of the conidia within the blossom interior. The 26.9% infection rate of the inoculated control pomegranates from week one in 2012 represents a 150% increase over the natural, background rate. In 2013, the 8.6% infection rate in the control was a considerable boost over the 1.2% background rate for week one (Table 1). For week two, the rate was effectively tripled by inoculating and bagging the blossoms at the optimum stage. All differences between the non-inoculated background and the inoculated control were significant at p<.05. A consequence of inoculating the blossoms was an increase in the number of abscised blossoms and ultimately fewer pomegranates at harvest from the initial 50 that were tagged for each replicate. We found that approximately 5 times the number of blossoms from the treatments and the control fell before harvest compared to the background blossoms (Table 2). For week one of 2013, 4.8% of the background blossoms did not make it to harvest. The range of the treatments and control for missing fruit was 22.8 to 28%. The same comparison for week two has 2.4% for the background versus a range of 8.8% to 13.6% for treatments and control.

In the process of examining pomegranates from the 2013 harvest, an extremely compelling case can be made for the stylar path of infection for black heart in pomegranates. Previously, it was suspected that punctures or cracks in the rind may be the entry point for *Alternaria* infection; large hemipteran insects such as the leaf-footed bug seemed like probable vectors of the disease. Consideration was also given to the pollen tube; as the conidia of *Alternaria alternata* are a fraction of the size of the pomegranate pollen grains. Perhaps a single conidium could travel the same path as the pollen grain to become a source of latent infection. After inspecting over 2000 pieces of fruit in the fall of 2013, it seems clear that infection by *Alternaria* slowly travels the length of the style and pollen tube through the mesocarp tissues and into the interior of the fruit. Infection may remain latent until growth conditions are more ideal for the fungus. Infection may also proceed at variable rates in different pieces of fruit and by different strains of the same *Alternaria* species. This has not been within the scope of any known experiments. There are instances of black heart where 100% of the fruit cavity is overcome with the dark fungus and other instances where perhaps only a small lesion, 1-5% of the cavity volume, is discolored without visible mycelia. This conclusion about the path of infection is based on the evidence that in every single instance of black heart, the darkened infected tissue of the pollen tube extended from the necrotic pistil to the outer edge of the mesocarp and into the locules. From this point, it is inferred that infection can proliferate within the internal cavity amidst the ripening arils. The extent of the infection may very well be dependent on the timing of the arrival of the infection slowly traveling the pollen tube. Whether or not a pomegranate becomes infected appears to be dependent upon the ability of the pathogen to infect and travel the course of the
pollen tube. Therefore, scrutiny was given to this tissue for each pomegranate that was inspected for black heart.

A stylar infection was determined to be significant if the dark coloration extended past the line that can be made at the base of the stylopodium (Figure 1). Of the 2118 fruit that were harvested from both weeks of the fungicide trial, 318 had significant stylar infections (15%). In every instance of the 160 fruit that had black heart infection, the extent of the stylar infection was significant. Not only was travel of infection significant, but for every instance of black heart infection, the extent of the pollen tube infection breached the endocarp and into the locules. For the additional 284 pomegranates inspected as part of the delayed inoculation trials, there were 87 stylar infections (31%) and 7 black heart infections. Again, in every instance of black heart infection there was a significant stylar infection. Examination of the stylar tubes revealed that the inoculation methods promoted greater travel of infection. For the week one inoculated control, 25% of the pomegranates had significant stylar infections; compared to the background rate of 8.8% (Table 1). For the week two inoculated control and background, the stylar infection rates were 26.7 % and 10.6 %, respectively. Given the successful induction a greater infection rates than those occurring naturally, inoculating and bagging the blossoms enhances the ability to assess the efficacy of fungicides sprayed at bloom.

Compared to the 8.6 % infection rate of the week one inoculated control, blossoms sprayed with fungicides yielded significantly fewer black heart infected fruit at harvest at p<.05 (Table 1 and Figure 2). Blossoms sprayed with Luna® Experience, Pristine®, or Polyoxin-D yielded black heart rates of 2.5, 2.6, and 2.6 percent, respectively. For week two, with a 17.6% infection rate from the inoculated control, the fungicides also significantly reduced black heart infected fruit at harvest. The rates for Luna, Pristine and Polyoxin-D treatments were 11, 8.9, and 12 percent respectively. When comparing the extent of stylar infections, the week one rates for blossoms sprayed with Pristine or Polyoxin-D were significantly lower than the 25% rate from the inoculated control at 7.3 and 11.3%, respectively. The 14.4 % rate in the Luna treatment was not significantly different at p<.05. For week two, with a 17.6% infection rate from the inoculated control, the fungicides also significantly reduced black heart infected fruit at harvest. The rates for Luna, Pristine and Polyoxin-D treatments were 11, 8.9, and 12 percent respectively. When comparing the extent of stylar infections, the week one rates for blossoms sprayed with Pristine or Polyoxin-D were significantly lower than the 25% rate from the inoculated control at 7.3 and 11.3%, respectively. The 14.4 % rate in the Luna treatment was not significantly different at p<.05. For week two, all treatments significantly reduced the extent of stylar infections (p<.05) compared to the 26.7% rate from the inoculated control. Luna, Pristine, and Polyoxin-D had rates of 12.3, 14.9, and 18.1 percent, respectively (Table 1).

Given the significantly higher drop rates for inoculated blossoms, whether or not the chemical treatment increases the frequency of blossom drop is important information. There were no significant differences between the treatments for the number of dropped blossoms at p>.05 (Table 2). There was also no effect from the treatment on the size of non-infected fruit at harvest. Additionally, the treatment did not affect the extent of the infection within the pomegranates. The average percentage of infected fruit volume ranged from 53.3 to 79.5%. Once an infection is initiated inside the pomegranate, the chemical treatment at bloom has no apparent effect on the rate of growth as the fruit approaches maturity.

The results from the trial to determine if inoculation timing had an effect on the infection rate were mostly in line with expectations except for some surprising results in the ‘+3 Week’ set of fruit (Table 3). The ‘0 Week’, or optimum bloom stage, yielded an infection rate of 8.6% (data from the inoculated control in main trial) compared to 0 and 1.0% for blossoms inoculated one and two weeks after ‘optimum’, respectively (Table 3). The surprise for the ‘+3 Week’ blossoms was the unexpected presence of black heart in 6.3% of the pomegranates. Analysis of variance
indicates that these differences are significant at p<.05; however, the black heart at week 0 was not significantly different from the disease incidence at +2 or +3 week inoculation. Given that only 100 blossoms were targeted within non-randomized rows, perhaps this was random variability or perhaps the trees within the +3 week row were more susceptible to infection of their blossoms by *Alternaria*. The alternative is that perhaps there is another window when the style and pollen tube are susceptible to the type of infection that can travel to the interior of the pomegranate.

**Pre-harvest sampling for latent infections by *B. cinerea***. Low and variable rates of latent *Botrytis* infection were observed from the plated sections of pomegranate calyces (Figure 3). It seems apparent that the 2320, B9-2 ranch had more latent infections for early samples, but rate of infections fell below the other two ranches for later sampling dates. This was a non-replicated survey with only 30 fruit samples per block and date, therefore, no statistical claims were likely to be made, but the intent was to see if a correlation could be drawn to the amount of post-harvest infection given the rates of latent infection. This will be addressed in the section that covers results for inspection of post-harvest pomegranates. In addition to finding latent infections for *Botrytis*, other fungal genera that were found on the plates include *Alternaria, Monilinia, Penicillium, Cladosporium, Botryosphaeria, Chaetomium, Rhizopus, Aureobasidium, Fusarium, Neurospora, Ptilidiella, Epicoccum, and Aspergillus* species. These fungi are common contaminants (acting as saprophytes) of plant surfaces in California fruit and nut crops. Of the most importance is the high incidence of *Alternaria* that was found in the pistillate tissue: 67% of the pistils that were plated resulted in growth of *Alternaria* spp. compared to a 4.4% rate for *Botrytis* from all three calyx tissues (pistil, stamens, and sepals). At the time of dissection and plating, it seems clear that all the pistils have necrotic tissue that was likely infected sometime after bloom. As noted with the inspection of pomegranates for black heart, the length of travel for these infections varies. It seems very likely that the majority of these infections of the style result from preliminary infection of the pistil by *Alternaria* spp.

**Gray mold after 3 and 5 months storage**. After 3 months in cold storage at 7.2°C and 85% RH, the symptoms of gray mold were apparent. As the crown was facing up, some beige to yellow discoloration around the calyx was visible as were more developed infections that had abundant mycelia and sporulation. The majority of the pomegranates were in good condition and appeared suitable for market. They did not appear to lose a significant amount of moisture. It did seem that the pomegranates from Block 2320, B-9 were somewhat drier than pomegranates from the other two blocks. Because of the pre-harvest sampling of the calyces, the infections of the crown region were of most importance, but stem infections were also noted (Figure 4 and Table 4). Pomegranates from Ranch/Blocks 2330/B10-A, 2320/B4-2, and 2320/B-9 had crown infection rates of 11.5, 12.5 and 2.6%, respectively. The 2320/B-9 ranch is the ranch that appeared to have lower latent infection rates closer to harvest (although higher rates further from harvest) and the fruit also appeared to have lost more water content in the rind. The same blocks had the following rates of crown infection at 5 months: 19.4, 20.3, and 3.6% respectively. Along with increasing rates of infection with extended storage, the rate of infections that initiated at the stem end of the pomegranate increased and took on a greater proportion of infections. At 3 months, 33.7% of infections initiated at the stem end. This increased to 72.2% by 5 months. By comparison, from 3 months storage to 5, the proportion of crown infections decreased from 68 to 43.8%. Another observation that may have value is that gray mold infections appear to
predominantly occur in the bottom layer of the boxes. At 3 months, 81.7% of the infections occurred on fruit packed on the bottom layer. At 5 months, this rate was 69%. The inference is that there is greater humidity on the bottom layer especially where moisture may be trapped between the crown and the plastic liner. This would foster ideal conditions for latent infection to emerge. Indeed, the majority of crown infections occur in the bottom layer: 91% at 3 months and 84.8 at 5 months.

**Efficacy of pre-harvest sprays at Kearney.** No significant differences were found between pomegranates receiving pre-harvest fungicide applications and the control at p<.05 (Figure 5). After inspections at 3 months and 5 months, there were fewer infections for pomegranates sprayed with Pristine at 1 week prior to harvest. However, there were more infections for pomegranates sprayed at 2 and 1 week prior to harvest. There was far too much variability to draw conclusions from the data, suggesting that the replication of trees to be treated needs to increase.

**Additional data obtained at end of experiments.** At the conclusion of the 5 month cold storage period, 30 non-infected pomegranates from each of the 3 blocks at Dudley Ridge and the block at KARE were dissected at the crown and sections from the calyx were plated as before. A higher rate of latent *Botrytis* infection was found. From Ranch/Blocks 2330/B10A, 2320/B4-2, and 2320/ B9-2 rates of calyx infections were 40, 60, and 10% respectively. Interestingly, Ranch 2320/B4-2 that had increasing levels of Botrytis latent infections during September, showed the highest incidence of gray mold after 5 month cold storage. Also of interest is that the low rate of latent infection for Ranch 2320/B9-2 is consistent with low incidence of gray mold in cold storage. The pomegranates sampled from Kearney had 43.3% latent infections after 5 months cold storage. Why these latent infections did not initiate infection after 5 months in cold storage is perhaps a question for a different study.

**Fungicide screening against Pilidilla granati and Monilia sp.** Growth in potato dextrose agar by isolates of both species tested was completely inhibited by Luna® Experience, Scholar®, and Switch® 62.5 (Figures 6 & 7). There was some growth of the isolates in the Merivon® amended media and there was total resistance of the isolates in the media amended with PhD®. In fact, the PhD amended media appeared to enhance the growth of the isolates acting as a nutrient source. Both Scholar and Switch contain the active ingredient fludioxinil which has been used to treat pomegranates prior to cold storage since 2001. Scholar contains 20.4% of this active ingredient and Switch 62.5 contains 25% fludioxinil and 37.5% cyprodinil. The active ingredient in PhD is polyoxin-D zinc salt which appears to have no activity against these fungi.

**Conclusions:**

From previous and mainly the 2013 studies conducted on black heart of pomegranate, we have determined that:
1) The infection pathway of *Alternaria alternata* and *Alternaria* spp. is almost certainly through the style and pollen tube to the locules and arils of the endocarp and that this infection is mostly likely to occur at the onset of bloom;
2) Inoculation at bloom induced the highest rate of black heart post-harvest;
3) Fungicide treatments significantly reduced black heart infection rates and the infection rates of the style and pollen tube; although there were no significant differences in efficacy among Luna® Experience, Pristine®, and Polyoxin-D;
4) Fungicide treatments did not affect the drop rate of blossoms or the sizing of fruit compared to those of the inoculated control.

From the 2013 studies conducted on gray mold of pomegranate, we have found that:
1) Within the three blocks studied there were no clear distinctions in the rates of latent *Botrytis* infection prior to harvest and therefore, no conclusions can be made about the relationship between the amount of latent infection and the amount of gray mold development during cold storage;
2) The crown of the pomegranate is more susceptible to gray mold after 3 months cold storage, but stem infection increases toward 5 months in cold storage;
3) The frequency of gray mold development appears to be much more problematic in the bottom layer of a 2-layered box and that the crowns of the pomegranate seem to be affected the most by being positioned against the plastic material;
4) Pre-harvest sprays within 2 weeks of harvest did not appear to have any significant effect reducing gray mold in cold storage (probably due to low infection rates).
5) In vitro fungicide screening revealed that *Pilidiella granati* and the putative *Monilia* species are totally inhibited by fungicides with active ingredient fludioxonil, including Scholar® and Switch®, as well as Luna® Experience. It is expected that after registration for pomegranate, the use of any of these fungicides may provide some benefits in managing diseases caused by *Pilidiella* and *Monilia* which are less prevalent on pomegranates.

Acknowledgements:

We thank Erik Wilkins, Norma Medrano, and the field crews from Paramount Farming Company for facilitating the field experiments at Dudley Ridge and for enabling the collection of significant crop loads for this experiment; and to Dave Morgan, Dan Felts, and Michael Luna from UC KARE Center for their assistance in the field and laboratory.

Literature Cited:

Ezra, D., Kirshner, B., Gat, T., and Kosto, I. 2013. Heart rot of pomegranate, when and how does the pathogen cause the disease. (Abstr.) The 3rd International Symposium on Pomegranate and Minor Mediterranean Climate Fruits (*in Greek*).


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**Tables and Figures:**

**Table 1.** Influence of fungicide treatments on 'Wonderful' pomegranate blossoms treated and inoculated at full bloom with *Alternaria alternata*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black heart³</td>
</tr>
</tbody>
</table>
**Table 2.** Additional parameters for pomegranates harvested from fungicide trials against black heart.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aborted fruit per rep. (out of 50)&lt;sup&gt;w&lt;/sup&gt;</th>
<th>Diameter (mm)</th>
<th>Mass (g)</th>
<th>Average extent of infection (%)&lt;sup&gt;x&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1&lt;sup&gt;y&lt;/sup&gt;</td>
<td>Week 2&lt;sup&gt;z&lt;/sup&gt;</td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>Pristine</td>
<td>12.6</td>
<td>6.2</td>
<td>79.6</td>
<td>82.7</td>
</tr>
<tr>
<td>Luna Exp.</td>
<td>14</td>
<td>6.8</td>
<td>80.4</td>
<td>84.8</td>
</tr>
<tr>
<td>Polyoxin-D</td>
<td>11.4</td>
<td>5.2</td>
<td>81.9</td>
<td>83.2</td>
</tr>
<tr>
<td>Inoc. control</td>
<td>12.2</td>
<td>4.4</td>
<td>81.2</td>
<td>84</td>
</tr>
<tr>
<td>Background</td>
<td>2.4</td>
<td>1.2</td>
<td>86.3</td>
<td>88.5</td>
</tr>
</tbody>
</table>

<sup>w</sup> Blossoms tagged on May 1<sup>y</sup> or 8<sup>th</sup> that fell shortly after inoculation or some time before harvest.

<sup>x</sup> Approximate percentage of the interior of infected pomegranate damaged by infection.

<sup>y</sup> Pomegranates resulting from blossoms inoculated on May 2<sup>nd</sup>.
<table>
<thead>
<tr>
<th>Week</th>
<th>Infections</th>
<th>Incidence</th>
<th>Total Infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1 Week</td>
<td>91</td>
<td>0.0</td>
<td>31.9</td>
</tr>
<tr>
<td>+2 Week</td>
<td>98</td>
<td>1.0</td>
<td>34.7</td>
</tr>
<tr>
<td>+3 Week</td>
<td>95</td>
<td>6.3</td>
<td>25.3</td>
</tr>
</tbody>
</table>

w Blossoms were tagged at full bloom for inoculation at later stage of development.

x Rate of black heart incidence determined by examination after September 19 harvest.

y Stylar infections assessed in conjunction with examination for black heart and deemed significant if the darkening of pollen tube extended past stylopodium.

z Data taken from the blossoms tagged for the background control in the first week of main trial.

Table 4. Distribution of infections by *Alternaria alternata* on untreated pomegranates harvested at Dudley Ridge and stored at 45° F and 85% RH.

<table>
<thead>
<tr>
<th>Infection Site</th>
<th>3 months</th>
<th>5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top layer</td>
<td>34</td>
<td>213</td>
</tr>
<tr>
<td>- Crown infection</td>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td>- Stem infection</td>
<td>24</td>
<td>179</td>
</tr>
<tr>
<td>Bottom layer</td>
<td>138</td>
<td>352</td>
</tr>
<tr>
<td>- Crown infection</td>
<td>105</td>
<td>190</td>
</tr>
<tr>
<td>- Stem infection</td>
<td>33</td>
<td>162</td>
</tr>
<tr>
<td>Total infections</td>
<td>169</td>
<td>511</td>
</tr>
<tr>
<td>Total fruit inspected</td>
<td>1517</td>
<td>1517</td>
</tr>
</tbody>
</table>

w Pomegranates stored at Kearney facility and examined at 3 and 5 month intervals. Fruit infected by 3 months were discarded to avoid spread (nesting) of infection with boxes.

x Location of infection within box and on fruit.

y Each box lined with #11 cupped liners.

z Total infections include fruit that had infections at both ends which were counted for both crown and stem infection categories.
Figure 1. Black heart infection initiated by significant stylar infection. Note that connection is made with locule of pomegranate.

Figure 2. Black heart incidence for pomegranates inoculated on May 2\textsuperscript{nd} (week one) and May 9\textsuperscript{th} (week two), 2013. Blossoms from separate blocks were sprayed with field rate applications on May 1 and May 8, 2013. The following morning they were inoculated with a 2x10\textsuperscript{4} conidia/ml spore suspension of \textit{Alternaria alternata} and bagged overnight. Rate of black heart incidence was determined by examination after September 19 harvest.
Figure 3. Incidence of latent infections by *Botrytis cinerea* from calyces of pomegranates harvested at pre-harvest intervals from three blocks at Dudley Ridge. For each date, 30 pieces of fruit were harvested from each block. A latent infection was determined if *Botrytis* was recovered from acidified potato dextrose agar plates with sterilized sepals, stamens, and pistils from each fruit.
Figure 4. Incidence and site of infection of gray mold caused by *Botrytis cinerea* for pomegranates harvested from Dudley Ridge and stored in cold storage at 7.2°C and 85% RH. Pomegranates were packed 22 per box with 2 layers and were inspected at 3 months and 5 months post-harvest. At the 3 month inspection, infected fruits were thrown out to avoid spread (nesting) of infections within boxes.
**Figure 5.** Efficacy of fungicide on gray mold caused by *Botrytis cinerea* for pomegranates treated with pre-harvest sprays at Kearney and stored in cold storage at 7.2°C and 85% RH. Pomegranates were packed 22 per box with 2 layers and were inspected at 3 months and 5 months post-harvest. At the 3 month inspection, infected fruits were thrown out to avoid spread (nesting) of infections within boxes.
Fig 6. Radial growth of three isolates each of Pilidiella granati (A) and Monilia sp. (B) in potato dextrose agar (PDA) amended with 10 µg/L active ingredient of Scholar®, Switch® 62.5, Merivon® and the unamended PDA control. Percent inhibition relative to control is in parentheses to right of columns. Measurements taken approximately 96 hours after 4 mm mycelial plugs were placed in center of media and plates incubated at room temperature or approximately 22°C.
Fig 7. Radial growth of three isolates each of *Pilidiella granati* (A) and *Monilia* sp. (B) in potato dextrose agar (PDA) amended with 10 µg/L active ingredient of Luna® Experience, PhD®, and the unamended PDA control. Percent inhibition relative to control is in parentheses to right of columns. Measurements taken approximately 66 hours after 4 mm mycelial plugs were placed in center of media and plates. *Pilidiella granati* plates were incubated at 30°C and the putative *Monilia* sp. plates were incubated at room temperature or approximately 22°C.