Basil Diseases Caused by *Peronospora belbahrii*, *Stemphylium* sp. and *Alternaria* sp. and Options for their Control

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I. Introduction

Basil production in Hawai‘i:

Hawai‘i’s agriculture industry is located in a unique environment, and therefore exotic agricultural crops have become important for export to the mainland USA. These include tropical ornamentals like orchids, tropical fruits including papaya, nuts, and other crops, such as basil , that cannot be grown in most of the mainland during the winter season (Abercrombie, 2011). Because of this unique winter growing season, crops like basil have become lucrative for growers in Hawai‘i, with crops exported to the mainland and Canada.

Diseases of basil in Hawai‘i:

During the winter months in Hawai‘i, sweet Italian (*Ocimum basilicum*) and Thai basil (O*cimum basilicum* var. *thyrsiflora*) are exported. For several years Hawai‘i-grown basil was in high demand in the mainland USA and Canada during the winter months. The industry made over $6 million per year until downy mildew entered Hawai‘i and severely impacted basil yields (Anonymous, 2012a). Downy mildew is more severe in the wet winter months when the crop is in its highest demand (Uyeda et al., 2012).

Another new disease of basil was reported in 2012. Apparently healthy, symptomless basil was packed and shipped to North America. When the basil arrived, black, gray, and water-soaked lesions began to develop and spread over the leaves, minutes after the boxes were opened. Researchers at CTAHR found mycelial growth within the black spots. *Stemphylium* and

Alternaria were isolated from these lesions (Uchida, unpublished). *Stemphylium* has not been reported as a pathogen on basil and Koch's postulates were needed to be to confirm pathogenicity. Similar studies with *Alternaria* also needed to be conducted, as it has been reported as a pathogen of basil (Garibaldi et al., 2011; Taba et al., 2009) and isolated in Hawai'i (Uchida, unpublished).

Some fungi, notably *Exserohilum rostratum* (Honda et al., 1978a), *Pleospora herbarum*, *Alternaria dauci* (Leach et al., 1966), *Stemphylium solani* (Sproston, 1971), and *Leptospaerulina trifolii* (Leach, 1972) require light for sporulation. Other species of the genus *Stemphylium* have been shown to have two stages in their asexual reproduction. First, there is an inductive stage where conidiophore production is induced by ultra violet light. The next stage is called the terminal phase, where conidia are formed. The terminal phase is inhibited by ultra violet light at 24°C. When pure cultures of these *Stemphylium* species are grown in vitro they must be grown first in light and then for a period in darkness to induce sporulation (Honda et al., 1978b; Leach, 1968; Metha, 2001).

Irradiation treatment for commercial produce export:

The shipment of fresh fruit and vegetables is regulated by the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS), which requires that all produce to be free of quarantine organisms when it is shipped to the mainland (Acord, 2002). All shipments to the mainland of 17 different fruits and 7 vegetables need to be treated to ensure that they are free of quarantine pests, while others like basil may be treated to

ensure that no hitchhiking pests will cause shipment rejection by APHIS inspectors (Follet, 2011). The majority of agricultural products are treated with chemical pesticides. However, some produce shipments are infected by other disease causing organisms that reduce quality and/or marketability of the produce (Fan et al., 2009). Thus, many diseased shipments of crops like Okinawan sweet potato, papaya, and basil are either rejected by agricultural inspectors upon arrival, or by consumers. The agriculture industry in Hawai‘i is motivated to develop methods for treatment of exported crops such as new chemicals, or alternatives to chemicals. One promising method of control that is presently being developed for new and current problems is phytosanitary irradiation (Hodgson, 1998). This promising technology has been adopted by the agricultural export industry in Hawai‘i. A good example of industry led efforts to disinfect Hawai‘i’s crops is the establishment of a new gamma irradiation facility in Kunia, Oahu in 2012.

The unit used to measure absorbed dose of irradiation in food is a gray (gry). One gray is the same as 100 rad (radiation absorbed dose), which is the old unit of measurement of absorbed dose of irradiation. These can be compared to the unit for human effective absorbed dose, Sv (Sieverts), where 1 Sv is equal to 1 gray. Human exposures for medical use can range from 0.004 mSv (millisieverts) for a dental bitewing to 57 mSv for a full PTCA (Percutaneous Transluminal Coronary Angioplasty) heart study (Anonymous, 2014a).

The Food and Drug Administration (FDA) has imposed limits of 1 kgry on plants to be sold as food for human consumption within the USA (Follett et al., 2009), which may be an issue if effective doses to control fungal rots are higher than this limit (Anonymous, 1986). However,

the FDA has made exemptions for specific food crops where the limit of 1 kgry is raised, such as spinach and iceberg lettuce which have a limit of 4 kgry (Anonymous, 2014d).

Commercial food irradiation can be accomplished with any of three irradiation options: electron beam, X-ray, or gamma ray irradiation. All three of these irradiation methods break down the DNA of the target organism, preventing its bio-chemical processes from functioning and reducing reproductive potential. Electron beam irradiation is achieved by placing food in a machine that projects electrons (sub-atom sized particles) towards a target. These particles penetrate the surface of the target, slowing as they enter. As the electron beams slow, energy is transferred to the target material. This type of irradiation is used as a surface sterilizer as penetration is low (Arthur et al., 2005).

X-ray and gamma ray irradiation employ energy rays, which include particle like photons that are directed towards a target, penetrate, and often pass through the target. Both X-rays and gamma rays have wavelengths of 10^{-9} to 10^{-13} meters, frequencies between 3^{13} and 3^{22} hertz, and energy levels of 10^{3} to 10^{7} electron volts. The difference between these two types of ionizing radiation are that the energy from X-rays comes from electrons, while gamma rays originate from atomic neutrons (Denny et al., 1999). This has implications for how they are employed for food irradiation, because this means that a radioactive substance, known as the "source" is required for gamma irradiation, while X-rays are produced by machines that energize electrons so that they release X-rays. X-rays can penetrate a meter or more into different materials and therefore can disinfect food more thoroughly than an electron beam

(Anonymous, 2014e). X-ray machines can be turned on and off, and the energy that the X-ray machines emit can be adjusted. Because some of the higher energy levels of X-ray irradiation can cause the substances that are irradiated to become radioactive, the FDA limits X-ray and electron beam irradiators to outputs of 4 Mev (Mega-electron volt) for food irradiation, which ensures that the food will not become radioactive (Anonymous, 2012b).

Gamma ray irradiation utilizes gamma rays in the same way that X-rays are used. They are emitted from radioactive metals (Anonymous, 2014e). Because the gamma irradiators require a source, the irradiation that they emit cannot be turned off and remains relatively constant, but gradually decreases as the source decays. Currently, the USDA approves the use of Cobalt 60 and Cesium 137 as source materials for gamma irradiators, which are deemed safe for food irradiation (Anonymous, 2014b). The gamma irradiator at Pa'ina Hawai'i LLC's Kunia facility is a Cobalt 60 Gray*Star Genesis II model. It features a single large pool of water into which bells that resemble luggage carts are lowered to receive the dose of irradiation from the cobalt 60 source (Fig. 1). The cobalt 60 source, or rods (called pencils) are located in the center of the pool (Fig. 1 blue), vertically positioned in a row, on the source rack (Fig. 2).

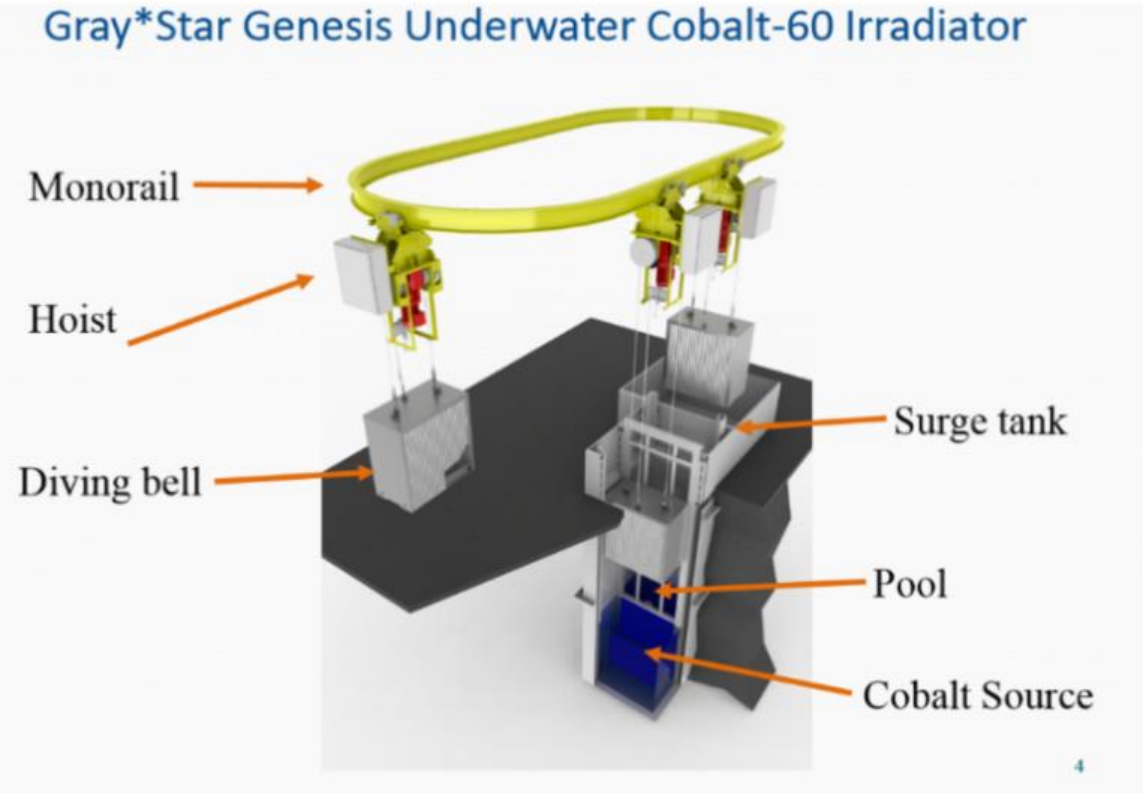

Figure 1. Diagram of a Gray*Star Genesis Underwater Cobalt 60 Irradiator (Wong, 2012b). Used with permission.

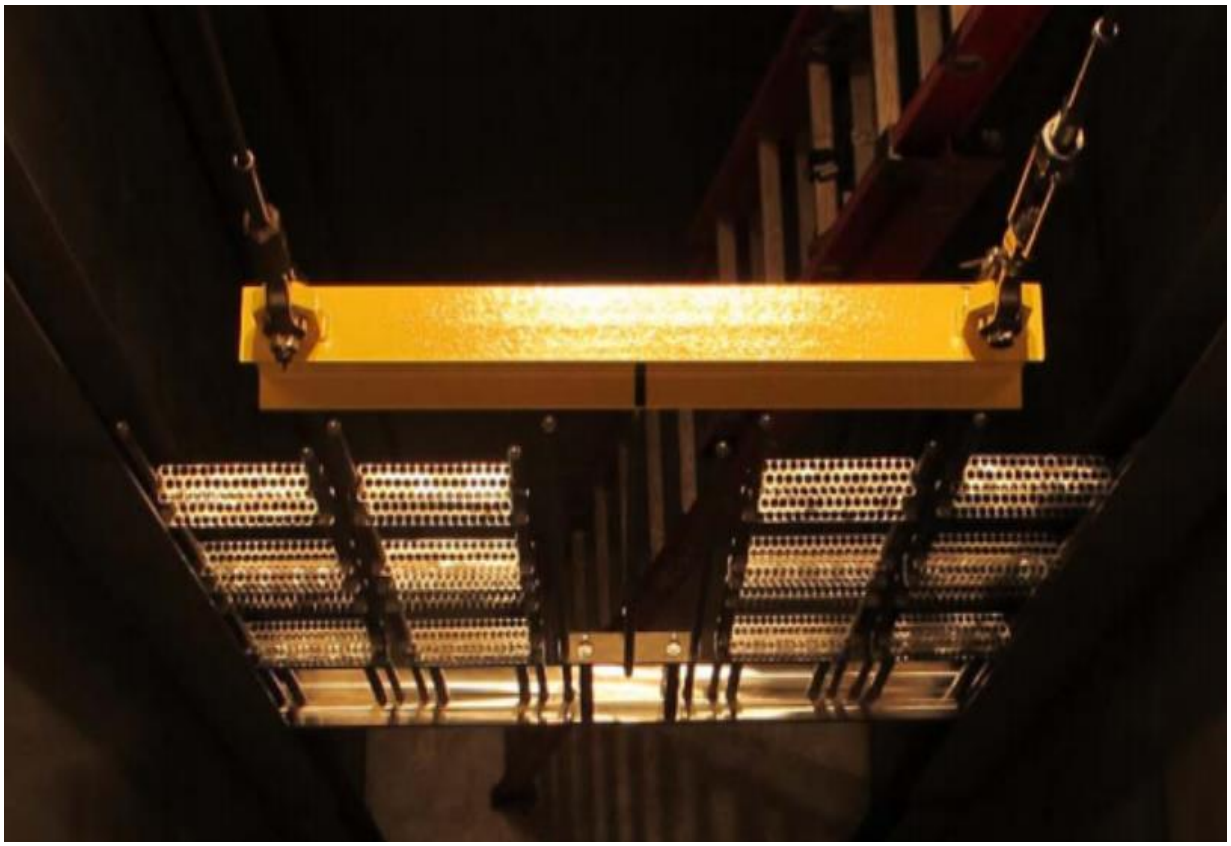

Figure 2. Cobalt 60 Source “pencils” or thin rods slide into the holes in the source holder below. They are oriented vertically on the source rack, which is oriented vertically in the pool (Wong, 2012a). Used with permission.

The bells loaded with produce typically begin the cycle from the entry/loading position 'A', on one side of the irradiation source pool (Fig. 3). Then the bell is moved and lowered into the pool for treatment at position 'B' (Fig. 3). To complete the treatment, the bell is lifted out of the pool, lowered into the other half of the pool at position 'C' (Fig. 3). When the treatment is complete, a mechanical winch lifts the bell back onto the rail system which is on a support structure fixed to the ground and moves the bell to the "clean" exit point. After the cargo has been unloaded, the rail system then loops the bell back to the loading station. This system is monitored by an "e-scan" dosimetry system, where alanine pellets are placed in the configuration of product to be irradiated (on the bell with the product) and then analyzed by a pellet reader and exported to a software program to ensure that the absorption of irradiation has reached the desired dose. Different configurations require different amounts of pellets, depending on the density of the treated product and the organization of the product on the carts.

Phytosanitary irradiation in Hawai'i:

In Hawai'i, several crops are commercially irradiated for export. Some of the crops approved for irradiation include but are not limited to: atemoya, banana, breadfruit, *Capsicum spp.*, carambola, citrus, *Cucurbita spp.*, dragonfruit, eggplant, guava, longan, lychee, mangosteen, moringa, papaya, pineapple, rambutan, sapodilla, sweet potato, tomato, and cowpea (Anonymous, 2014c). These crops are approved for a generic quarantine dose of 400 gry for control of all insects and 150 gry in some cases (Follet et al., 2011).

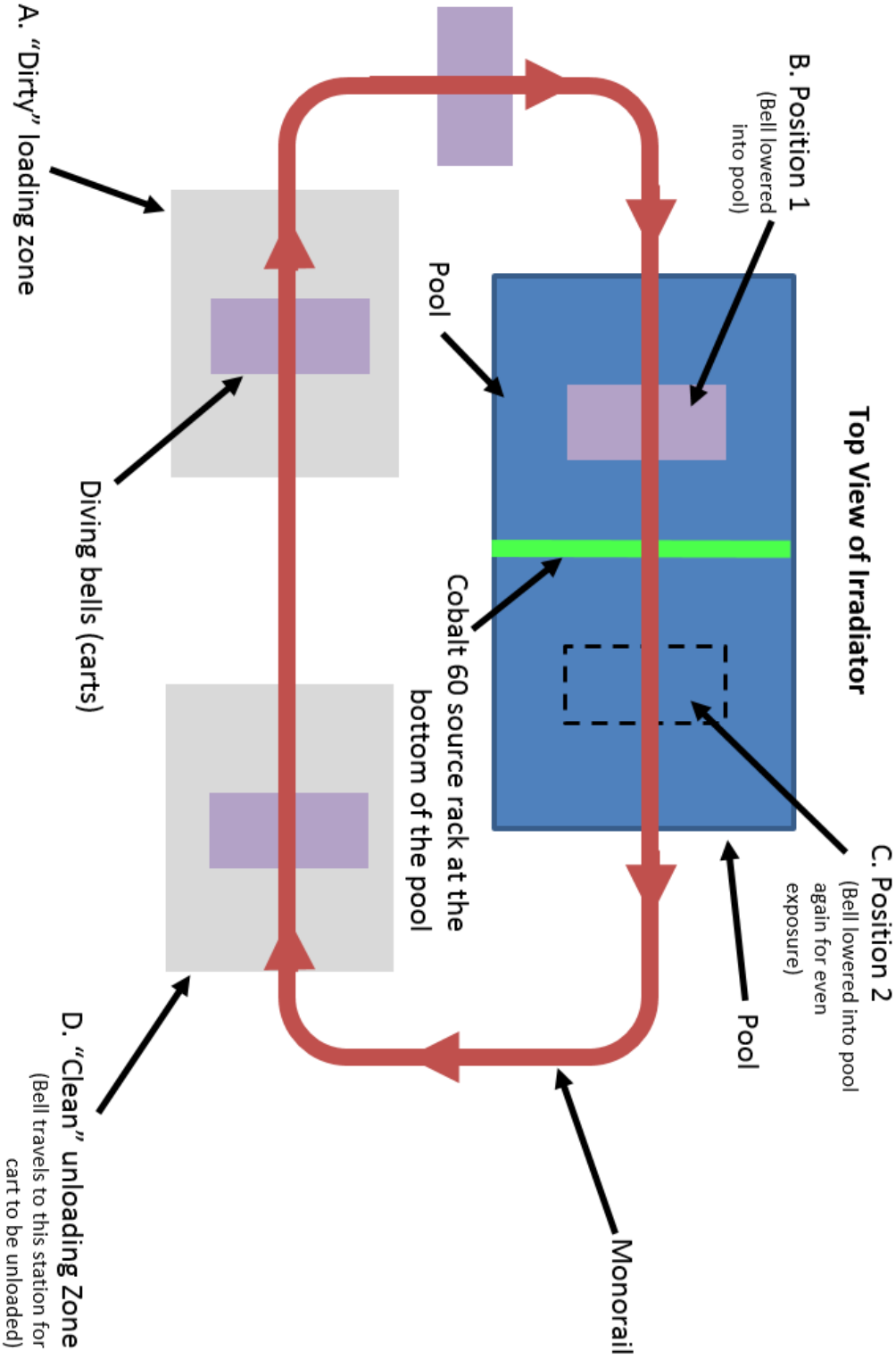

Figure 3. Diagram of irradiator and it's operation

A more pronounced need for effective post-harvest treatments exists in Hawai‘i compared to the US mainland, as exports need to be shipped further to reach export markets. Currently, fungicides are used to control *Peronospora belbahrii* the causal agent of basil downy mildew, although this pathogen is known to develop resistance to fungicides relatively quickly (Hausbeck et al., 2012). Therefore, other methods of control for this disease are needed. Tentative information from Pa'ina Hawai‘i LLC. shows that fresh basil has a relatively high tolerance to gamma irradiation when compared to other vegetables, and can withstand high doses before electrolyte leaking becomes an issue. Doses of 1 kgry have been applied to basil with no harmful effects observed by the operators of Pa'ina Hawai‘i LLC. The most common symptom associated with over-irradiation of leafy greens is electrolyte leaking. Symptoms of electrolyte leaking, are leaf sogginess, wilted appearance, and blackening due to the deterioration of cell walls (Fan et al., 2008). In leafy greens, the dose at which electrolyte leaking can become apparent can range from anywhere between doses just above 1 kgry to approximately 4 kgry (Fan et al., 2008).

Chemical Control:

For pathogens of basil, several chemical fungicides have been tested for efficacy on downy mildew, caused by *Peronospora belbahrii*. These include Quadris® (a.i. azoxystrobin), Fosphite® (a.i. mono and dipotassium salts of Phosphorous acid), Trilogy® (a.i. clarified hydrophobic extract of neem oil), and Regalia® (a.i. extract of *Reynoutria sachalinesis*). In a field trial, Quadris® and Fosphite® had the best results for reducing visible disease of downy mildew

and improving basil yield (Kawate et al., 2013). Hence, these two fungicides may be the most effective pair to use in a fungicide rotation program for basil to avoid fungicide resistance. Chemicals previously tested in these field trials will be compared to other potential chemical treatments in greenhouse trials. Chemicals previously tested in these field trials will be compared to other potential chemical treatments in greenhouse trials. Fungicide resistance of *P. belbahrii* to the few products now labeled for use on basil has created the need to evaluate new products that are coming onto the market (Hausbeck et al., 2012). Revus®, a new fungicide released into the U.S. market in 2014 needs to be field tested against *P. belbahrii* in Hawai'i.

II. Objectives

The first objective is isolation and culture of potential pathogens of basil on the island of Oahu that are associated with the gray spot disease. The next objective is to determine the pathogenicity of the *Stemphylium* and *Alternaria* isolates that were found in association with the gray spot of basil disease. The third objective is the identification of the *Stemphylium* and *Alternaria* isolates to species level.

Chemical fungicides will be evaluated for efficacy in reducing disease caused by *Peronospora belbahrii*. Then, irradiation will be evaluated for efficacy in reducing disease caused by *Peronospora belbahrii*, *Stemphylium*, and *Alternaria*. Symptom development of both uninfected basil, basil infected with *Peronospora belbahrii*, and basil infected with both *Stemphylium* and *Alternaria* will also be evaluated after irradiation treatment.

III. Materials and Methods

Isolation and Culture:

Fungal pathogens were isolated from harvested (market maturity) Italian sweet basil. Fungal isolations were made by cutting the tissue interface between healthy tissue and diseased lesions in approximately 1 cm sized sections, surface sterilizing sections in a 10% NaOCl solution for about 5 seconds, and placing the sterilized tissue on water agar. Single hyphal tips were cut from the fungi that grew out and were grown on 10% V8 agar. Single spore cultures were obtained by using a glass needle to transfer single spores to new V8 agar plates. One culture was chosen as the stock culture, from which more cultures were made. These plates were grown out as pure cultures for 7 days. If spores did not grow naturally, cultures were placed in complete darkness for 24 hours to induce sporulation.

10% V8 agar was made by adding 0.384 Fisher brand $CaCO_3$ to 160 ml of V8 brand vegetable juice and shaking in a glass bottle. A 50 ml aliquot was added to a 1 liter flask, along with 5.5 g USBC brand agar powder and 440 ml of de-ionized H_2O. An aluminum foil cover was placed over the flask and the flask was autoclaved for 20 minutes. The autoclaved agar was cooled at room temperature for 20 minutes. The agar was stirred until homogenous. The agar was then poured into petri dishes and allowed to solidify. Cultures were grown on 10% V8 agar at 25°C under constant fluorescent light. *Stemphylium* cultures were grown under fluorescent light for 6 days and then 1 day in complete darkness to induce uniform sporulation. *Alternaria* cultures were grown in fluorescent light for 7 days.

Pathogenicity Tests:

Pathogenicity tests on basil were conducted for *Stemphylium* and *Alternaria*. *Stemphylium* culture 401B.1 and *Alternaria* culture 905 were used for the pathogenicity tests. For both fungi, spores were harvested by spraying cultures with a 1:1000 Tween 20 (Polyoxyethylene sorbitan monolaurate) solution. Spore concentration was estimated by counting a subsample with a hemacytometer and inoculum was adjusted to 10^5 spores/ml. Inoculum was sprayed to drip onto three plants of Thai basil and three of Italian sweet basil for each test. Three control plants of each cultivar were sprayed to drip with the Tween 20 solution. All of the plants were incubated in clear plastic bags at 25°C in the shade for 24 hours at 100% humidity. For one set of pathogenicity tests, plants were maintained in the greenhouse and monitored daily for symptom development for 14 days after the incubation period. For a second set of pathogenicity tests, two days after inoculation, plants were cut, placed in clear plastic bags, and packed into a box, and refrigerated at 17° C for 2 days to mimic shipping conditions. After this cooling period, the basil was removed from the boxes and bags, and observed at room temperature for 1 hour. Symptoms were recorded, and compared to those exhibited by the basil from growers in the spring of 2013. Symptom scoring was conducted with a presence or absence scale (Anonymous, 2011). Presence of any symptoms included: greying or blackening of patches of leaf tissues, water soaking, wetness and viscosity, and wilting. If any of these symptoms were observed, it was recorded that there was a presence of disease. If no symptoms were observed, it was recorded that the disease was absent in the sample.

Species Identification:

Molecular identification

After pathogenicity of *Stemphylium* and *Alternaria* isolates were established, both were identified to species. Identification was accomplished by two means. First, molecular methods were used to provide a tentative species identification. Second, these results were compared with morphological descriptions and compared to the isolates tested.

The molecular identification was based on the ITS region. Seven day old cultures of *Stemphylium* and *Alternaria* were grown on 10% V8 agar. *Stemphylium* (400B.1) was grown for 6 days in light, and one day in darkness, while *Alterneria* (905) was grown in light for 7 days. The following steps were identical for both fungi. A 0.5 g fungal sample was scraped from the top of the culture dish, ground with a mortar and pestle with enough Buffer AP1 from the Qiagen DNeasy® Plant Mini Kit to keep the fungal material from adhering to the mortar. Then, the remainder of the 400 µl of extraction buffer was added along with 4 µl of RNase A. The suspension was vortexed and incubated in a water bath at 65° C for 10 minutes. Then 130 µl of Buffer P3 was added to the solution, mixed with a pipette, and incubated for 5 min on ice. The lysate was then centrifuged for 5 min at 20,000 x g (14,000 rpm). The lysate was then pipetted into a Qishredder™ mini spin column, contained inside a 2 µl collection tube. These were then centrifuged for 2 min at 20,000 x g. The liquid that flowed through the Qishredder™ mini spin column was transferred to a new tube. The remaining liquid was measured, 150% of this volume was calculated and Buffer Aw1 was added at this volume to the remaining solution, and mixed by pipetting. A 650 µl aliquot of the mixture was pippeted into a DNeasy® mini spin

column in a fresh 2µl collection tube and centrifuged at 6,000 x g (8,000 rpm) for 1 min. The flow through was discarded and this step repeated with the remainder of the mixture. The DNeasy® Mini spin column was placed into a new 2µl collection tube. A 500 µl unit of Buffer AW2 was added and the column was spun at 20,000 x g for 2 min. The spin column was transferred to a new 2µl microcentrifuge tube. A 60 µl unit of Buffer AE was added and the column was incubated at room temperature for 5 min. This incubation was followed by centrifuging for 1 min at 6,000 x g. A 60 µl unit of Buffer AE was added and the column was incubated at room temperature for 5 min, followed by centrifuging for 1 min at 6,000 x g.

The end product from this DNeasy® procedure was used as template DNA for a PCR reaction to amplify the ITS region of the fungus for sequencing. Primers for the ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) and ITS5 (5´-GGAAGTA AAAGTCGTAACAAGG-3´) region were used with an Eppendorf thermocycler, with the following program: 94° C for 3 min followed by 35 cycles of 94° C for 1 min, 55° C for 1 min, 72° C for 2 min and a final extension at 72° C for 5 min (Metha, 2001). Ten µl 2x GoTaq® mastermix was mixed with 1 µl template DNA from the DNeasy® product, 0.5 µl ITS4 forward primer and 0.5 µl ITS5 reverse primer, and 8 µl sterile H_2O for the PCR reaction. The results of the PCR products were then electrophoresed and evaluated for fragment length and intensity next to a Thermo-Scientific Gene Ruler 100bp-1kbp ladder. The PCR product was cleaned with an ExoSAP-IT® kit by mixing 5 µl PCR product with 2 µl ExoSAP-IT®, with a pipet in a clean 1.5 ml microcentrifuge tube. The microcentrifuge tube and contents were incubated for 15 min at 37° C followed immediately by incubating at 80° C for 15 minutes. A 1 µl unit of cleaned PCR product was then mixed by pipet with 0.5 µl ITS4 forward

primer and 6.5 µl sterile H_2O. These DNA, for both *Alterneria* and *Stemphylium*, were submitted to Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) at the University of Hawai'i at Manoa for sequencing. Results from ASGPB were copied and a "blastn" search was used to find the closest match. The closest matches were used to compare isolates with morphological descriptions.

Morphological identification

Morphology of *Stemphylium* was compared to the reference sources of *Stemphylium lycopersici* (Ellis, 1971; Nishi et al., 2009; Gannibal, 2011), *Stemphylium solani* (Ellis, 1971; Gannibal, 2011), and *Stemphylium vesicarium* (Ellis, 1971; Ichikawa et al., 1994). *Stemphylium* culture 400B.1 was grown on 10% V8 agar under light for 6 days, followed by 24 hours of darkness. Spores and conidiophores were placed on a microscope slide and measured under a light microscope at a magnification of 400x.

Morphological comparisons of *Stemphylium* were made by measuring the length of conidiophores and the width of the swollen apical cell of the conidiophore. The length, width, number of transverse septations, and longitudinal septations of spores were measured. The shape of the apical region of the conidia, the surface texture of the conidia and conidiophores, culture color, conidiophore and conidia color were also recorded. One hundred conidiophores and conidia were measured and compared to the reference materials.

Morphology of *Alternaria* was compared to morphological descriptions in reference materials (Ellis, 1971; Simmons, 2007; Garibaldi et al., 2011; Taba et al., 2009). Morphological comparisons of *Alternaria* were conducted by measuring the length of conidiophores, and the width of the swollen apical cell of the conidiophore. *Alternaria* culture number 905 was grown on V8 agar for 7 days under light, after which time, conidiophores and conidia were collected, placed on a slide and measured under a light microscope at a magnification of 400X. The length, width, beak width, number of transverse septations, and longitudinal septations were also measured. The surface textures of the conidia and conidiophores, the number of conidia per chain, culture color, and color of conidia and conidiophores were also measured. One hundred conidiophores and conidia were measured and averages were then compared to the reference materials.

Chemical fungicide trial:

A randomized complete block designed greenhouse experiment was conducted for the chemical fungicide evaluation. The experimental unit was a single potted Italian basil plant with three replications per treatment. Treatments were a control spray of H_2O with Latron B-1956™ spreader sticker (modified phthalic glycerol alkyd resin), a soil drench of Quadris® (azoxystrobin), and a foliar spray of Revus® (mandipropamid) with Latron B-1956™. The treatments were organized into three blocks, by date of trial.

Italian basil plants were made from clean cuttings from Fat Law Farms. Cuttings at similar stages of development were then inoculated with downy mildew. Plants with similar

infection levels of downy mildew were randomly assigned to a treatment, and were organized randomly on a greenhouse bench (www.random.org).The number of infected leaves was defined as leaves with visible sporulation. Infected leaves and total leaves were counted and percentages recorded before treatment. The plants were sprayed to drip for the control and Revus® treatments while a soil drench was performed for the Quadris® treatment. For the control spray, 250 ml of H_2O was mixed with 0.3125 ml of Latron B-1956™ and approximately 85 ml of solution was sprayed per plant. For the Quadris® treatment 1.17 ml of Quadris® was mixed with 30 ml of H_2O per plant. The concentration of the final treatment was 250 ml of H_2O was mixed with 0.3125 ml of Latron B-1956™ and 36.6 ml of Revus®. An aproximately 85 ml aliquot of the Revus® suspension was sprayed onto each plant, covering all surfaces.

Observations were taken weekly, and the ratio of infected leaves to total leaves per plant were recorded. An ANOVA and mean separations were conducted using SAS 9.3 statistical software (SAS Institute, Inc., Cary, NC). Infection rate data was sine-transformed prior to ANOVA, and means were separated by Tukeys t-test (α=0.05) wherever appropriate.

Irradiation efficacy:

Healthy basil was harvested From Fat Law Farms, placed in perforated plastic bags, and placed in a cardboard box which was taped shut. The basil was irradiated to determine the maximum dose that the basil can tolerate without losing marketability. The boxes were places on the center of the irradiator cart. One dosimeter was placed on the top center of each box to

ensure accurate absorbed dose. The rates used were 1 kgry, 2 kgry, 7 kgry and 10 kgry. The treated boxed-basil and an equal number of untreated boxed-basil were then placed in a 17°C incubator. The basil was evaluated subjectively by 1) visual means directly after irradiation on a two tier scale of light or heavy symptoms and 2) compared with untreated controls every 24 hours for 3 days post irradiation.

Symptom development on *Stemphylium*, *Alternaria, and Peronospora belbahrii* infected basil:

Field infected basil was collected from a commercial farm located in the central part of the island of Oahu. Samples were bagged in open ended plastic bags, placed in cardboard boxes and irradiated at 1 kgry and 2 kgry, with a set of basil used as untreated controls. The basil was placed in perforated plastic bags in a closed cardboard box and incubated for 3 days at 17° C. After 3 days, the basil was removed from the incubator and observed for symptom development, and the process repeated three times. The basil was rated on a three tier scale: 1-symptomless or few small necrotic flecks, 2- moderate amounts of necrotic flecks and spots covering approximately half the leaf surface, 3- necrotic spots that have grown together to cover over 50% of the leaf surface and the leaf being limp, slimy, and water-soaked. Symptoms were recorded and isolation of *Stemphylium* and *Alternaria* from the symptomatic basil was attempted from approximately 20 leaf sports per treatment.

Field basil infected with *Peronospora belbahrii* was harvested, bagged and boxed as described. The basil was irradiated at 1 kgry and 2 kgry. To determine if the downy mildew was still alive, leaves from both treatments were placed directly on the top of healthy leaves of

living plants in separate sets for each treatment. Another set of plants was similarly inoculated with untreated infected leaves. The inoculated plants were observed for 10 days with symptoms and sporulation noted for 3, 5, 7, and 9 days following inoculation. On the tenth day, the basil plants were evaluated on a 4 tier scale for symptom severity:

1=symptomless and no signs present,
2=mild symptoms (such as vein delimited chlorosis) and no signs present,
3=moderate symptoms (such as extensive chlorosis and necrotic flecks), no signs present,
4=heavy symptoms and/or signs present (such as sporulation and large blight-like lesions).

Also, field infected basil with mild to moderate symptoms (chlorosis but no necrosis) with sporulation was scored, bagged, treated with irradiation and then boxed and placed in a 17°C incubator to simulate shipping conditions. This basil was evaluated again after 72 hours. For this process a different four tier scale was used:

1=symptomless and no signs present
2=mild symptoms/signs with no necrosis (such as sporulation, vein delimited chlorosis)
3=moderate symptoms/signs with moderate amounts of necrosis (such as sporulation necrotic patches within chlorotic areas)
4=severe symptoms/signs with extensive necrosis (sporulation, blighted leaves, extensive necrotic lesions, absence of any green leaf tissue).

IV. Results

Pathogenicity:

During the greenhouse *Stemphylium* pathogenicity tests no leaf spots developed. In the tests that simulated shipping conditions of cut basil, a “melting symptom” characterized by dark color, wet tissue was commonly observed after the 3 day incubation period. The leaf tissue of the basil became soft, water-soaked and somewhat translucent within 10 minutes of removal from the 17°C incubator and being placed in room temperature (Figure 4 A and B).

Figure 4. *Stemphylium* pathogenicity test with comparisons between the control and inoculated plants A. Typical control plant B. Close-up of a typical inoculated plant leaf.

In general, the older leaves were more likely to become symptomatic. The Italian basil showed more severe symptoms than the Thai basil. On both basil cultivars, the control plants had zero instances of leaf symptoms after being removed from the incubator, and in each trial at 67% of all inoculated plants showed symptoms.

The *Alternaria* pathogenicity tests resulted in no leaf spot development. In the tests with refrigerated cut basil, a softening, blackening/water-soaking, translucent and slimy symptom was commonly observed after the 3 day incubation period. Within 10 minutes of being removed from the refrigerator, plant became symptomatic, as shown in Figure 5 A and B. On both basil cultivars, the control plants had no of leaf symptoms after removal from the incubator. In each trial at least 67% of all inoculated plants showed symptoms.

Figure 5. *Alternaria* pathogenicity test on basil A. Typical control plant B. Typical inoculated plant.

Identification:

The “blastn” sequence analysis on the *Stemphylium* showed three possible matches (Table 1). The first match with 100% sequence homology was *S. lycopersici* with a 90% query cover. The second hit was *S. solani* with 99% sequence homology and a 93% query cover, and another match for *S. solani* with a lower homology but larger coverage of the same ITS sequence at 98% sequence homology and 98% query cover. The third match was *S. vesicarium* with 98% sequence homology and a 96% query cover.

The morphological characteristics of *Stemphylium* culture 400B.1, were compared to published measurements (Figs. 6 -8 and Tables 2-4). The most defining feature is the apex shape. The apex shape “conical” refers to a sharply angled point giving the apex a resemblance to a cartoon water droplet. The “blunt or rounded” apex refers to an apex with no sharp angles with a rounded appearance.

The closest morphological match to culture 400B.1 was *S. vescicarium* (Tables 2-4). The single most important morphological factor is the blunt or rounded apex, followed by the length and width of the spores. These correlate closest to *S. vescicarium* (Ellis, 1971; Gannibal, 2012; Ichikawa et al., 1994; Nishi et al., 2009). The “blastn” search and morphology that culture 400B.1 demonstrates the species to be *S. vescicarium*. Two factors are important in for sequence comparison. Sequence homology is the percentage of nucleotides that are identical between the two sequences. Query coverage is the overlap of the range of nucleutides compared. The higher the query coverage, the more likely that any mismatched nucleotides will be shown in the sequence homology. The blastn query cover for *S. lycopersici* and *S. solani* was

Table 1. CLUSTAL W (1.83) multiple sequence alignment of *Stemphylium* isolates from GenBank compared with isolate 400B.1

Stemphylium Species ID	Sequence Data

```
solani|JF913268.1       ----------------------CCTGCGGAGGGATCATTACA—CAATA
lycopersici|JF417683.1  --------------------GAACCTGCGGAGGGATCATTACA—CAATA
vesicarium|GU065719.1   GTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACA—CAATA
400B.1                 ----------------------------CGGAGTACTGATCGAGGTCAAAG
                                                   *****   * **      ***
solani|JF913268.1       TGAAAGCGGGCTGGGACCTTAC-----TTCGGTGAGGGCT---CCAGCTT
lycopersici|JF417683.1  TGAAAGCGGGCTGGGACCTTAC-----TTCGGTGAGGGCT---CCAGCTT
vesicarium|GU065719.1   TGAAAGCGGGTTGGGACCTCAC-----CTCGGTGAGGGCT---CCAGCTT
400B.1                 TTAAAAAAAATAGGGTCTTGATGGATGCTCAACCAAGGCTGATTCAAAGT
                       * ***       *** * * *       **    * ****    **   *
solani|JF913268.1       GTCTGAATTATTCA----CCCATGTCTTTTGCGC--ACTTCT--TGTTTC
lycopersici|JF417683.1  GTCTGAATTATTCA----CCCATGTCTTTTGCGC--ACTTCT--TGTTTC
vesicarium|GU065719.1   GTCTGAATTATTCA----CCCATGTCTTTTGCGC--ACTTCT--TGTTTC
400B.1                 GCAAGAATTGTGCTGCGCTCCGAAACCAGTAGGTCGGCTGCCAATGATTT
                       *   ***** * *      **    *   *  *    ** *   ** **
solani|JF913268.1       CTGGGCGGGT-TCGCCCGCCACCAG--GACCAA-ACCA--TAAACCTTTT
lycopersici|JF417683.1  CTGGGCGGGT-TCGCCCGCCACCAG--GACCAA-ACCA--TAAACCTTTT
vesicarium|GU065719.1   CTGGGCGGGT-TCGCCCGCCACCAG--GACCAA-ACCA--TAAACCTTTT
400B.1                 TAAGGCGAGTCTCGTGAGAGACAAGACGCCCAACACCAAGCAAAGC---T
                          **** ** ***   *  ** **  * **** ****   *** *   *
solani|JF913268.1       TGTAATTGCAATCAGCGTCAGTAAACAA-TGTAATTATTACAACTTTCAA
lycopersici|JF417683.1  TGTAATTGCAATCAGCGTCAGTAAACAA-TGTAATTATTACAACTTTCAA
vesicarium|GU065719.1   TGTAATTGCAATCAGCGTCAGTAAACAA-TGTAATTATTACAACTTTCAA
400B.1                 TGAGGGTACAAATGACGCTCG--AACAGGCATGCCCTTTGGAATACCAAA
                       **    * ***    **   *  ****    *     **  **     **
solani|JF913268.1       CAACGGATCTCTTGGTTCTGGCAT-CGATGAA-----GAACGCAGCGAAA
lycopersici|JF417683.1  CAACGGATCTCTTGGTTCTGGCAT-CGATGAA-----GAACGCAGCGAAA
vesicarium|GU065719.1   CAACGGATCTCTTGGTTCTGGCAT-CGATGAA-----GAACGCAGCGAAA
400B.1                 GGGCGCAA-TGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATT
                          ** *  * *  ****    ** ******      ***  * ** *
solani|JF913268.1       TGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
lycopersici|JF417683.1  TGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
vesicarium|GU065719.1   TGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
400B.1                 CACACTACGTATCGCATTTCGCTGCGTTCT-----TCATCGA-TGCCAGA
                         *  ******  *    * ** *  ***      ******* *    **
solani|JF913268.1       ACGCACAT-TGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCG--AGC
lycopersici|JF417683.1  ACGCACAT-TGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCG--AGC
vesicarium|GU065719.1   ACGCACAT-TGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCG--AGC
400B.1                 ACCAAGAGATCCGTTGTTGAAAGTTGTAATAATTACAT-TGTTTACTGAC
                       **  * *  * **   **     **  **     *    ****      *
solani|JF913268.1       GTCATTTGTACCCTCAA----------GCTTTG--CTTGGTGTTGGGCGT
lycopersici|JF417683.1  GTCATTTGTACCCTCAA----------GCTTTG--CTTGGTGTTGGGCGT
vesicarium|GU065719.1   GTCATTTGTACCCTCAA----------GCTTTG--CTTGGTGTTGGGCGT
400B.1                 GCTGATTGCAATTACAAAAAGGTTTATGGTTTGGTCCTGGTGGCGGGCGA
                       *    *** *    ***          * ****  * *****  *****
solani|JF913268.1       CTT-GTCTCTCA--CGAGACTCGCCTTAAAATCATTGG---------CAG
lycopersici|JF417683.1  CTT-GTCTCTCA--CGAGACTCGCCTTAAAATCATTGG---------CAG
vesicarium|GU065719.1   CTTTGTCTCTCA--CGAGACTCGCCTTAAAATGATTGG---------CAG
400B.1                 ACCCGCCCAGGAAACAAGAAGTGCGCAAAAGACATGGGTGAATAATTCAG
```

```
                              * *    *  * ***   **   ***   ** **         ***
solani|JF913268.1             CCGACCT---------ACTGGTTTCGGAGCGCAGCACAATTCTTGCACTT
lycopersici|JF417683.1        CCGACCT---------ACTGGTTTCGGAGCGCAGCACAATTCTTGCACTT
vesicarium|GU065719.1         CCGACCT---------ACTGGTTTCGGAGCGCAGCACAATTCTTGCACTT
400B.1                        ACAAGCTGGAGCCCTCACCGAAGTAAGGTCCCAGC-CCGCTTTCATATTG
                              * * **         ** *   *  *  * **** *   * *   * *
solani|JF913268.1             TG-AATCAGCCTT-----GGTTGAGCATCCATCAAGACCCTATT---TTT
lycopersici|JF417683.1        TG-AATCAGCCTT-----GGTTGAGCATCCATCAAGACCCTATT---TTT
vesicarium|GU065719.1         TG-AATCAGCCTT-----GGTTGAGCATCCATCAAGACCACATT---TTT
400B.1                        TGTAATGATCCCTCCGCAGGTT---CACCTACCGAGACATTGTTACGTTT
                              ** *** * ** *     ****   ** * * * ****    **   ***
solani|JF913268.1             TTTAACTTTTGACCTCG------
lycopersici|JF417683.1        TTTAACT----------------
vesicarium|GU065719.1         TTCAACTTTTGACCTCGGATCAG
400B.1                        TTTAACTTCCA------------
                              ** ****
```

- Sequence data unavailable
* Sequence similarity among all species isolates

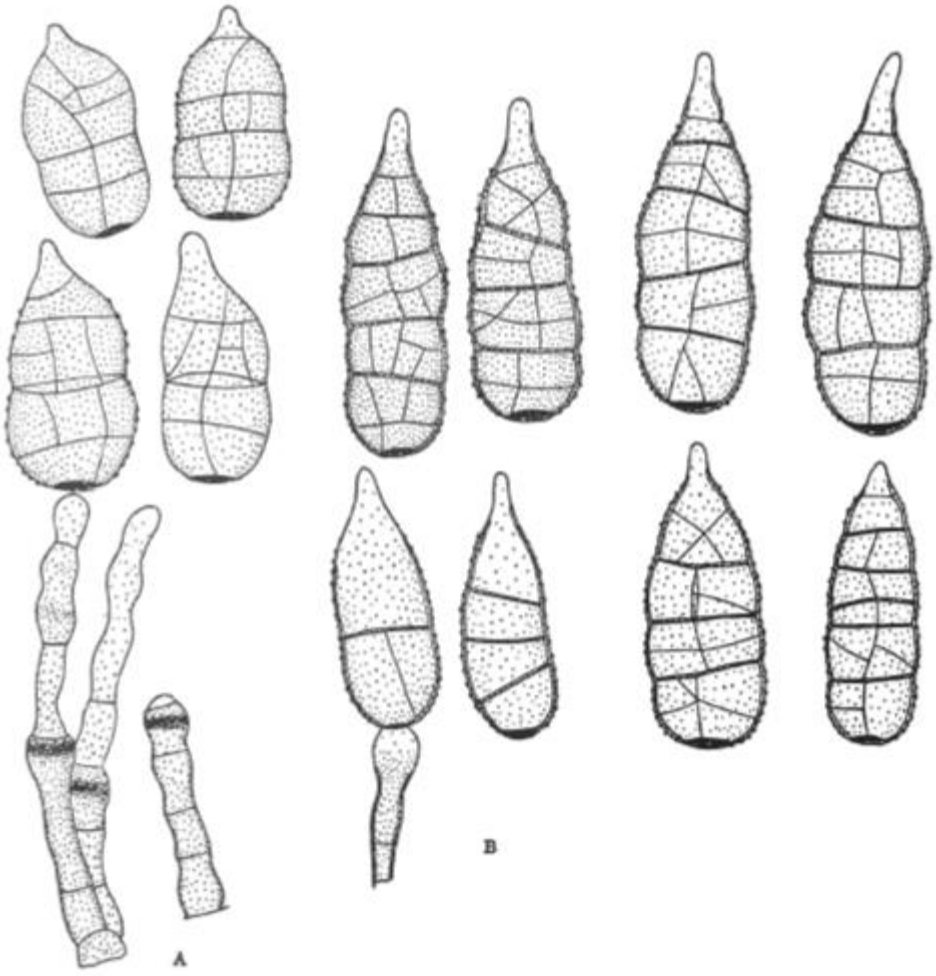

Figure 6. Conical apex of *Stemphylium solani* and *Stemphylium lycopersici. Stemphylium* species: A, *solani;* B, *lycopersici* (x 650) (Ellis, 1971).

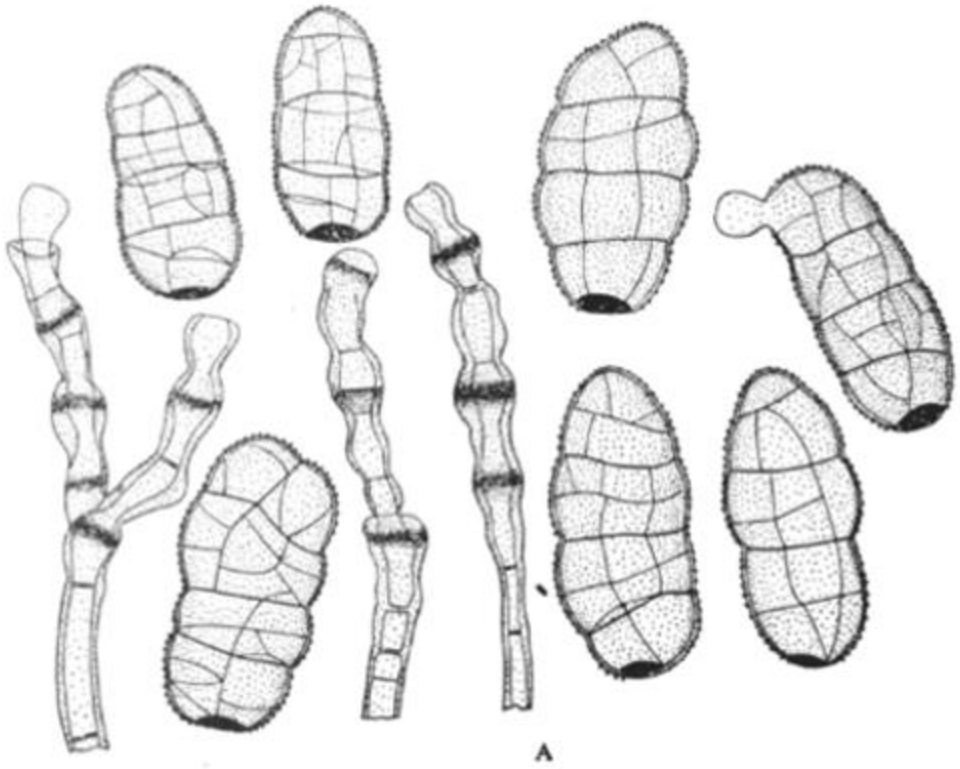

Figure 7. Blunt, rounded apex of *Stemphylium vesicarium*. *Stemphylium* species: A, *vesicarium* (x 650) (Ellis, 1971).

Table 2. Morphological measurements of *Stemphylium vesicarium*

	Source	a	b	Culture 400B.1
Conidiophore	Length	70	~70 or 32 -270*	28.8 - 850 (avg 233)
	Apical cell width	8 - 11	8 - 11 or 6 - 8	1.5 - 7.5 (avg 6.2)
Conidia	Length	20 - 50	20 - 50 or 40 - 55	17.5 - 50 (avg 36.7)
	Width	15 - 26	15 - 26 or 15 - 18	10 - 20 (avg 14.9)
	Transverse septations	6	~6 or~7**	3 - 8 (avg 5.8)
	Longitudinal septations	several	3	1 - 3 (avg 1.6)
	Smooth or Verrucose	verrucose	verrucose	Smooth or minutely verrucose
	Apex shape	Blunt and rounded	Blunt and rounded	Blunt and rounded

* "~" not clarified in source but assumed to be "approximately"

** Information not clarified in source

a. (Ellis, 1971)

b. (Ichikawa et al., 1994)

Table 3. Morphological Measurements of *Stemphylium lycopersici*

	Source →	a	b	c	Culture 400B.1
Conidiophore	Length	N/A*	140	30 - 151.3	28.8 - 850 (avg 233)
	Apical cell width	N/A	8 - 10	6	1.5 - 7.5 (avg 6.2)
Conidia	Length	50 - 74	50 - 74	31.3 - 70	17.5 - 50.3 (avg 36.7)
	Width	16 - 23	16 - 23	12.5 - 20	10 - 20 (avg 14.9)
	Transverse septations	5 - 8	1 - 8	4 - 16	3 - 8 (avg 5.8)
	Longitudinal septations	N/A	several	several	1 - 3 (avg 1.6)
	Smooth or Verrucose	Smooth or minutely verrucose	Smooth or minutely verrucose	Smooth or minutely verrucose	Smooth or minutely verrucose
	Apex shape	conical	Conical	Conical	Blunt and rounded

* "N/A" means not available

a. (Gannibal, 2012)

b. (Ellis, 1971)

c. (Nishi et al., 2009)

Table 4. Morphological Measurements of *Stemphylium solani*

	Source →	a	b	Culture 400B.1
Conidiophore	Length	N/A*	200	28.8 - 850 (avg 233)
	Apical cell width	N/A	8 - 10	1.5 - 7.5 (avg 6.2)
Conidia	Length	35 - 55	35 – 55	17.5 - 50.3 (avg 36.7)
	Width	18 - 28	18 - 28	10 - 20 (avg 14.9)
	Transverse septations	3 - 6	3 - 6	3 - 8 (avg 5.8)
	Longitudinal septations	N/A	several	1 - 3 (avg 1.6)
	Smooth or Verrucose	N/A	Smooth or minutely verrucose	Smooth or minutely verrucose
	Apex shape	Conical	Conical	Blunt and rounded

* "N/A" means not available

a. (Gannibal, 2012)

b. (Ellis, 1971)

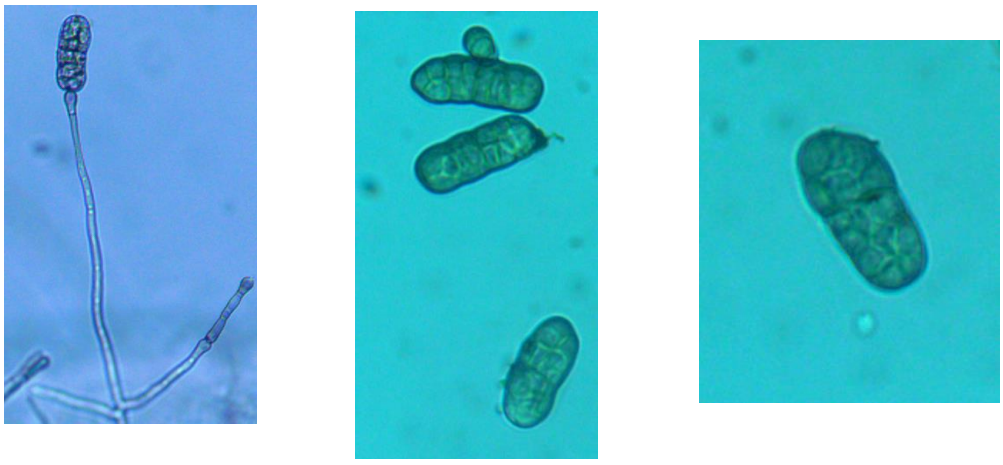

Figure 8. Photographs of spores from *Stemphylium* culture 400B.1 (magnification of photos are 200x (left), 400x (center), and 600x (right])).

much lower than that of *S. vescicarium* in spite of having a higher sequence homology. The relatively low query coverage for *S. lycopersici* and *S. solani* compared to *S. vescicarium* may explain the high sequence homology, as there is not as many base pairs being compared, and therefore less chance for a difference to occur.

Using the "blastn" search for *Alternaria*, the closest match was *A. alternata* with 99% sequence homology and a 97% query cover. There were no other close matches from the "blastn" search.

Alternaria isolate 905 was used for pathogenicity tests and the sequencing. The "blastn" search revealed one potential match to species based on this sequence. The sequence comparison for *A. alternata* compared to culture 905 showed sequence homology of 99% with a 97% query coverage (Table 5). A total of three isolates of *Alternaria* were recovered from diseased basil tissue, and they all had the same morphological characteristics as culture 905.

The morphology of culture 905 was compared to that of several morphological identification reference isolates (Table 6). Considering the diversity in spore sizes of the *Alternaria*, color of cultures, and number of conidia per chain, it was concluded that isolate 905 was *A. alternata* (Ellis, 1971; Garibaldi et al., 2011; Simons, 2007; Taba et al., 2009). The sequence data and the blastn search also found a high similarity between this fungus and *Alternaria alternata*.

Table 5. CLUSTAL W (1.83) multiple sequence alignment of *Alternaria* isolates from GenBank compared to isolate 905.

Alternaria Species ID	Sequence data
alternata|JN107734.1	----------GG--AAGGTAAAAAACGTAACAAGGTCTCCGTAGGTGAAC
905	CTGATCTACTGATCGAGGTCAAAGTTGAAAAAAGG-CTTAATGGATG---
	* **** *** * ** **** ** * * **
alternata|JN107734.1	CTGCGGAGGGATCATTACACAAATATGAAGGCGGGCTGGA--ACCTCTCG
905	CTA------GACCTTTGCTGATAGA-GAGTGCGACTTGTGCTGCGCTCCG
	** ** * ** * * * * ** *** ** * **
alternata|JN107734.1	GGGTTA-CAGCCTTGCTG--AATTATTC--ACCCTTGTCTTTTGCGTACT
905	AAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCTCCAGCAAAGC
	* ** * **** ***** * * * **** ** *
alternata|JN107734.1	T----------CTTGTTTCC--------TTGGTGGGTTCGCCCACCAC
905	TAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGC
	* * ** * * **** * * *
alternata|JN107734.1	TAGGACAAACATAAACCTTTTGTAAT---------TGCAATCAGCGTCAG
905	TCGAACAGGCAT--GCCCTTTGGAATACCAAAGGGCGCAATGTGCGTT--
	* * *** *** ** **** *** ***** ****
alternata|JN107734.1	TAACAAATTAATAATT-----------ACAACTTTCA--AC--AACGGAT
905	CAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCAT
	** * * ** *** *** * ** ** * ** **
alternata|JN107734.1	CTC-TTG-GTTCTGGCATCGATGA-AGAACGCAGCGAAATGCGATAAGTA
905	TTCGCTGCGTTCTT-CATCGATGCCAGAAC-CAA-GAGATCCGTT--GT-
	** ** ***** ******** ***** ** ** ** ** * **
alternata|JN107734.1	GTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG--AACGCACATT
905	-TGAAAGTTGTA-----------ATTATTAATTTGTTACTGACGCTGATT
	** * *** * ** ** * * ** **** ***
alternata|JN107734.1	GCGCCCTTTGGTATTCCAAAGGGC--ATGCCTGTTCGAGCGTCATTTGTA
905	GCA---------ATTACAAAAGGTTTATGTTTGTCCTAGTGGTGGGCGAA
	** *** **** ** *** *** * ** * * *
alternata|JN107734.1	CCCTC-----AAGC------TTTGCTTGGTGTT-GGGCGTCTTGTCTCTA
905	CCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTC--A
	*** * ** * * ** *** * * * *
alternata|JN107734.1	GCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTT
905	GCAAGGCTGTAACCCCG------AGAGGTTC-CAGCCCGCCTTCATATTT
	** **** * * ** ** ** ***** **** * ***
alternata|JN107734.1	CGGAGCGCAGCA--CAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCC
905	----GTGTAATGATCCCTCCGCAGGTTC-ACCTACGGAGACCTTG-----
	* * * * **** ** * * * ** ** *
alternata|JN107734.1	ATTAAGCCTTTTTTCAACTTTGACCTC
905	-TTACGTTTTTTT---ACTTC--CCA-
	*** * ***** **** **

- Sequence data unavailable
* Sequence similarity among all species isolates

Table 6. Morphological Measurements of *Alternaria alternata*

	Source →	a	b	c	d	Culture 905
Conidiophore	Length	21.3-50.9 (avg 34.9)	≤50	N/A*	~50**	11.5-177.5 (avg 57.6)
	Width	3.6-4.8 (avg 4.3)	3-6	N/A	N/A	2.5-5.8 (avg 4.6)
	Smooth or Verrucose	N/A	smooth	N/A	N/A	smooth
Conidia	Length	12.6-47.2 (avg 30.7)	20-63 (avg 37)	23.7-73.4	13-43	15-65 (avg 36.1)
	Width	9.7-16.5 (avg 12.2)	9-18 (avg 13)	8.8-15	8-14	7.5-15 (avg 10.8)
	Transverse septations	1-7	≤8	3-7	2-10	1-9 (avg 4.7)
	Longitudinal septations	1-3	Usually several	0-4	0-2	0-2 (avg 0.7)
	Apex width	3.7-4.6 (avg 4.4)	2-5	N/A	N/A	2-7.5 (avg 3.8)

* N/A" means not available

** "~" not clarified in source but assumed to be "approximately"

a. (Taba et al., 2009)

b. (Ellis, 1971)

c. (Garibaldi et al., 2011)

d. (Simons, 2007)

Chemical fungicide trial:

The data from the fungicide trial fit the assumptions of the ANOVA for comparison in percent change of infected leaves from pre-treatment until 1 week after treatment. In this period, Revus® was the most effective treatment based on the Tukeys means separation performed, reducing disease levels by 27.3% after 2 weeks (Table 7).

Table 7. Tukeys means separation for week 1-week 2 of the chemical fungicide trial. Revus® is shown to be significantly more effective than the control and Quadris®, which showed no significant difference to each other.

trt	N	Mean	Tukey Grouping
Quadris®	9	6.667	A
Control	9	4.0	A
Revus®	9	-27.333	B

Means with the same letter are not significantly different.

In comparison, the data from pre-treatment until 2 weeks after treatment showed only slight differences among treatments (P=0.0477).

A significant difference between Quadris® and Revus® was found (Table 8). No difference was found between the control and Revus® or between the control and Quadris®. Week 2 and week 3 visual comparisons show the mean amount of infected leaves in the Revus® treatment were lower than control and Quadris® treatments, but the means were not statistically different (Fig. 9).

Table 8. Tukeys means separation for week 1-week 3. The table shows that the only significant difference found was between Revus® and Quadris®.

Trt comaprison	Difference between means	Simultaneous 95% confidence limits
rev - cont	0.3242	-0.3249 0.9732
rev - quad	0.7621	0.0682 1.4559 ***
cont - rev	-0.3242	-0.9732 0.3249
cont - quad	0.4379	-0.2559 1.1317
quad - rev	-0.7621	-1.4559 -0.0682 ***
quad - cont	-0.4379	-1.1317 0.2559

Comparisons significant at the 0.05 level are indicated by ***

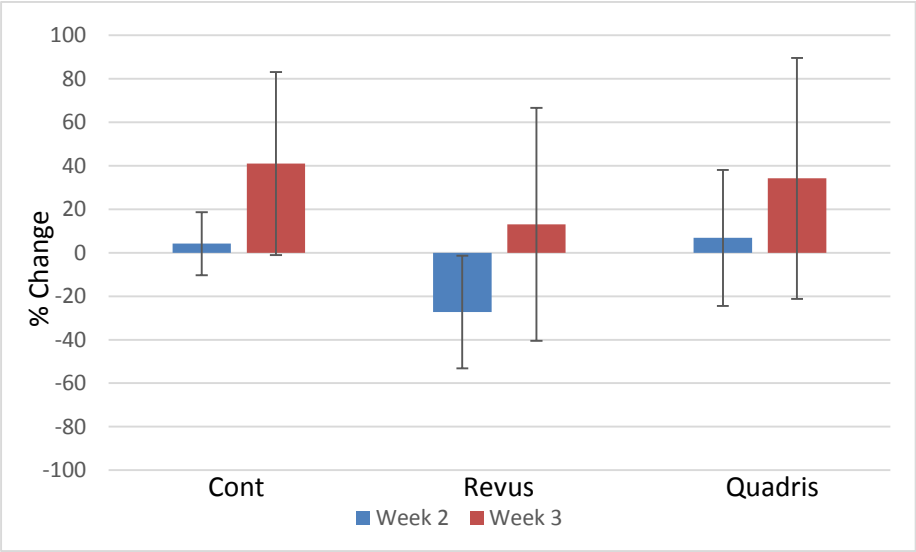

Figure 9. Fungicide efficacy comparing week 2 and week 3 observations of % infected leaves of basil infected with *Peronospora belbahrii*. Error bars are standard deviation of treatments.

Irradiation Efficacy:

Field infected basil irradiated at 1 or 2 kgry, and the untreated control showed inconsistent symptoms after refrigeration. Both *Stemphylium* and *Alternaria* were isolated from field infected plants at each dose (control, 1 kgry, 2 kgry). Typical symptoms after irradiation were for the plant to wilt and to turn black (Figs. 10-12).

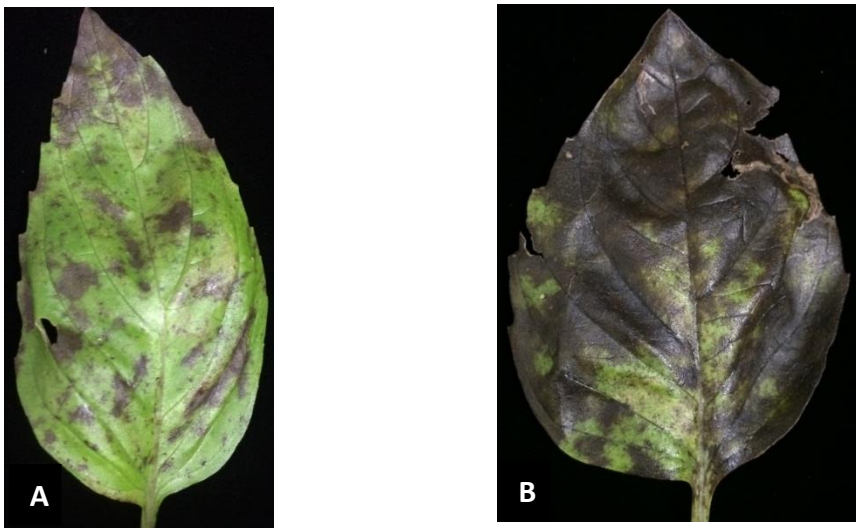

Figure 10. Typical symptom range of untreated *Stemphylium* and *Alternaria* field infected basil (A) at one on the visual scale, and (B) at three on the visual scale.

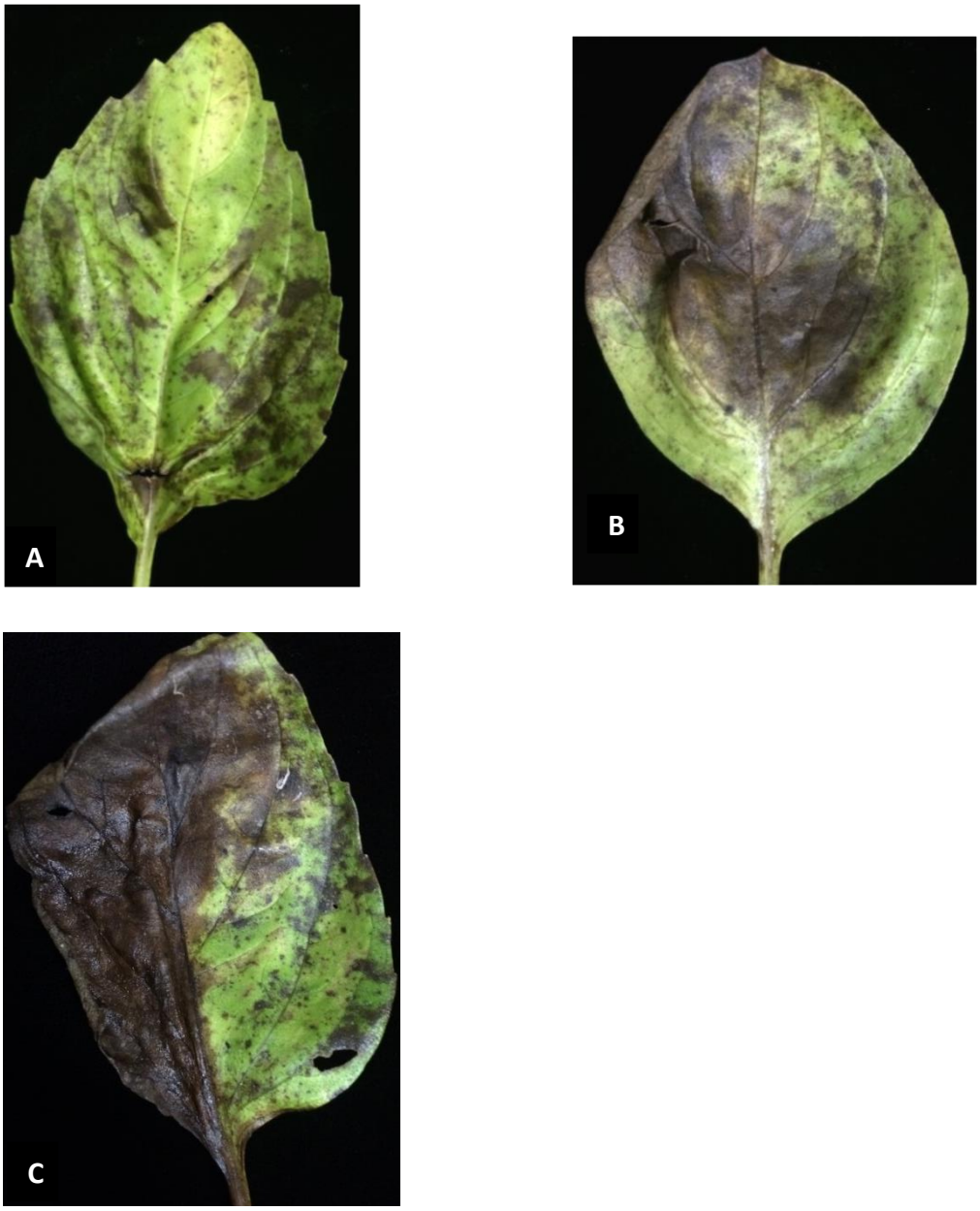

Figure 11. Typical symptom range from 1-3 on the visual scale of 1 kgry treated *Stemphylium* and *Alternaria* field infected basil. (A) 1 (B) 2 and (C) 3 on the visual scale.

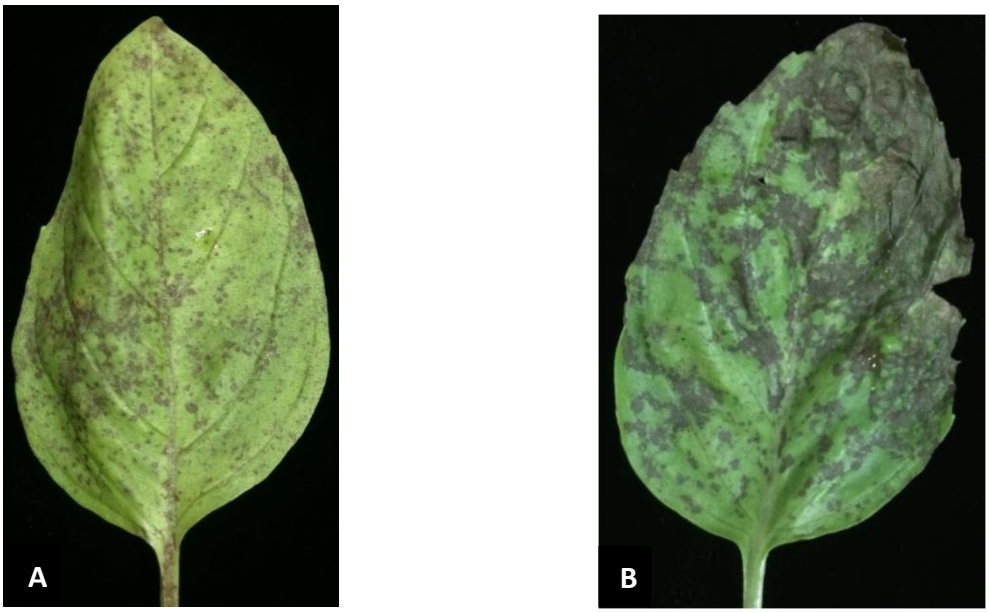

Figure 12. Typical symptoms from 2 kgry *Stemphylium* and *Alternaria* field infected basil (A) 1 (B) 2 on the visual scale.

The amount of leaves with a disease rating of 1 was highest for the untreated controls in the first and second trials. The treatment with the highest disease rating was the 1 kgry treatment for the first trial, and the 2 kgry treatment for the second trial. The third trial was inconclusive as no disease developed.

Peronospora belbahrii spores remained viable, regardless of irradiation treatment. All of the healthy basil inoculated with the treated (1 kgry and 2 kgry) and untreated control infected basil plants repeatedly became infected at the same time, displaying similar severity of symptoms and signs. After 10 days, the heathy basil inoculated with untreated infected basil, 1 kgry treated infected basil, and 2 kgry infected basil repeatedly had a disease rating of 4. The 1 and 2 kgry irradiation treatments had no effect on the viability of *Peronospora belbahrii* spores and did not delay disease progress.

Peronospora belbahrii infected basil treated at 1 kgry and 2 kgry had large necrotic areas covering areas that had been only symptomatic or showing limited signs when the plants were bagged. The symptoms on the untreated controls advanced only to necrotic flecks on chlorotic and sporulating patches of tissue during the same period. The untreated controls had a disease rating of 2-mild symptoms/signs with no necrosis The 1 kgry treated basil had a disease rating of 4, severe symptoms/signs with extensive necrosis. The 2 kgry treated basil had a disease rating of 4 with severe symptoms/signs and extensive necrosis. *Peronospora belbahrii* infection symptoms on basil after 72 hours of refrigeration, post irradiation (Fig. 13).

Figure 13. Symptoms of untreated *Peronospora belbahrii* field infected basil. (A) A 2 rating on the visual scale. (B) A 4 on the visual scale. (C) A 3 on the visual scale (D) A 4 on the visual scale.

V. Conclusion/Discussion

This is the first report of *Stemphylium* as a pathogen of basil. The post-harvest disease that *Stemphylium* causes make it a noteworthy pathogen for basil that is packed and shipped across long distances and over long periods of time. Local markets may not experience the same disease issues as this *Stemphylium* requires darkness and/or refrigeration over the span of 2 days for disease development.

Alternaria alternata has been shown to be a pathogen of basil in Japan and Italy (Garibaldi et al., 2011; Taba et al., 2009). However, this is the first time that *A. alternata* has been reported in Hawai‘i and the USA on basil. This is extremely important to the basil industry in the United States, since *Alternaria alternata* is especially severe during the winter months when Hawai‘i produces high volumes of basil for export to the US mainland. The post-harvest symptoms observed in the *Stemphylium* and *Alternaria* simulated shipping tests, were consistent with the symptoms reported on basil after refrigerated shipments arrived on the US mainland markets in March 2013. Pathogenic *Alternaria* species are often but not always characterized by long apical beaks (Cotty et al., 1984; Garber et al., 2011). Isolate 905 has a short apical beak and symptoms are not evident until the basil has been harvested and handled, so it is possible that this organism is strictly a postharvest pathogen.

Stemphylium vescicarium and *A. alternata* infecting basil without the expression of symptoms is not unique. *Stemphylium vescicarium* infects grasses, and completes its lifecycle without causing symptom development on those hosts (Rossi et al., 2005). *Alternaria alternata*

infects *Gossypium hirsutum* (cotton) without causing symptoms (Bashan, 1994). It is unknown how these pathogens gain energy from their hosts without causing symptoms. In, this case leaf spots were sometimes induced with different environmental conditions, such as temperature fluctuations and changes in lighting. Refrigeration and darkness may play roles in the symptom onset of *S. vescicarium* infected cut basil. To reinforce this argument, other *Alternaria* species have been reported to infect cotton and potato, (*Solanum tuberosum*), and yet produce no symptoms until environmental conditions such as temperature and amount of available light triggered symptom development (Bashan, 1994; Leiminger et al., 2015).

The most effective chemical treatment available for use on basil to manage basil downy mildew is Revus®. However, the effects of Revus® are short lived. It offers protection for 1 to 2 weeks, after which, the plants must be retreated. Efficacy of Quadris® was statistically indistinguishable from a water spray in its ability to control basil downy mildew when applied to diseased basil. This highlights the importance of a fungicide resistance management program, application of fungicides before widespread infection, and a rotation of different fungicides to manage basil downy mildew. The lack of efficacy in this study compared with its success in a previous study in 2013 also highlights the need for additional effective chemicals to come onto the market soon, to ensure an effective resistance management rotation can be employed by the basil growers in Hawai‘i for the management of basil downy mildew (Kawate et al., 2013).

Irradiation treatments above 2 kgry harm healthy basil. Exposures over 2 kgry would not be feasible treatments. Two out of three irradiation trials on plants showing symptoms associated with “gray spot of basil” had similar symptoms to basil plants inoculated with

Stemphylium and *Alternaria*. The reason for delay in symptom development is may be due to fungicide applications in the field, field environmental conditions at the time of basil harvest, or because the fungi react to cold temperatures and/or darkness in a way to activates their pathogenicity.

In tests of basil infected with *Stemphylium* and *Alternaria*, results show the pathogens are clearly able to survive the irradiation treatments. Although symptom severity is reduced at progressively higher treatment rates, irradiation did not significantly or consistently improve the quality of the basil or eliminate the pathogen on infected samples.

Peronospora belbahrii spores treated at 1 kgry and 2 kgry were able to infect healthy basil at the same rate and severity as untreated spores. Also, irradiation treatments of downy mildew infected basil increase all symptom severity dramatically. Therefore, irradiation treatments of downy mildew infected basil are not effective in delivering a marketable product and will not stop the spread of *Peronospora belbahrii*.

For future research, it may be useful to test the effects of modified atmosphere packaging in tandem with irradiation for management of post-harvest diseases of basil. However, this research shows that currently, the most effective methods of basil downy mildew control are in the field. Proper integrated pest management (IPM) programs can reduce inoculum levels of *Peronospora belbahrii*, *Stemphylium vescicarium*, and *Alternaria alternata* and curb disease in the field. Imported propagative materials (which may be infected with pathogenic *Stemphylium*, *Alternaria*, and *Peronospora belbahrii*), and propagation of basil by

cuttings may ensure that the next season's crop is already infected before planting. Use of seeds to propagate basil, strict quarantine, chemical rotations, increased crop spacing, and use of non-overhead irrigation systems may be the best management practices currently available for basil growers in Hawai‘i.

VI. References

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Chapter 2: Gamm irradiation's effects on *Stemphylium* and *Alternaria* growth

I. Introduction

Irradiation treatments are currently being used for three major purposes on food products. One purpose is disinfection of food from human pathogenic bacteria such *Escherichia coli* and *Salmonella* (Anonymous, 2012b). Another purpose is to eliminate quarantine pests from plant materials before shipping to a location that is free of that pest (Hodgson, 1998). One more purpose is to extend shelf life of some food products (Fan et al., 2009). Shelf life reduction can in many cases be attributed to different fungi and bacteria that grow on and in food before it is eaten, spoiling the food (Betts, 2006a; Pitt, 2006; Samelis, 2006; Williams, 2006). Bacterial tolerances to irradiation have been well researched and are common treatments to reduce harmful bacteria counts for such items as ground beef or shellfish (Anonymous, 2012b; Baylis, 2006; Betts, 2006b; Fan et al., 2009; Fan et al., 2008; Liao, 2006; McClure, 2006).

Fungal tolerances to irradiation have been less well researched, and the literature available on some of these fungi are in conflict with each other (Table 9). Tolerances of two genera *Stemphylium* and *Alternaria* are variable, depending on what source is referenced. Salama et al. (1977) claim that *Stemphylium* is killed at 5 kgry while Geweely et al. (2006) claim that the "lethal dose" is 3 kgry. Salama et al. claim that *Alternaria* spores are still able to germinate at 5 kgry while Geweely et al. claim that the "lethal" dose is 3 kgry. The term "lethal dose" is not clarified by Geweely et al., which adds to the confusion, although it may be supposed that all spores are dead at the "lethal" dose.

Table 9: Fungi controlled by irradiation available in the literature

Fungal genus	Dose	Effect
Stemphylium	500 krad (5 kgry)	Reduction in spore germination from 52.3% to 0% (Salama et al., 1977)
	3 kgry	"lethal dose"* (Geweely et al., 2006)
Alterneria	500 krad (5 kgry)	Reduction in spore germination from 54.3% to 5.8% (Salama et al., 1977)
	3 kgry	"lethal dose"* (Geweely et al., 2006)

*not clarified or defined in source, but implies that all spores are killed.

The disparities demonstrate that a clearly defined, objective approach needs to be taken to resolve the discrepancy of radiation tolerance of these fungi. A model needs to be created for further studies.

II. Objectives

Stemphylium and *Alternaria*'s tolerances to irradiation needs to be determined. This will be done in two ways. Spore germination after treatment of different doses of irradiation will be measured to determine spore viability. Also, mycelial growth will be measured to determine the ability of the filamentous part of the fungi to grow post treatment.

III. Materials and Methods

Effects of irradiation on the pathogens *Alternaria* and *Stemphylium* were determined by spore germination, radial mycelial growth, and survival in the host. For irradiation treatments

using petri dishes, the petri dishes were stacked along the X axis of the irradiation cart, and centered along the y axis (Fig. 14). The stack of petri dishes was three long and two high (three spore plates and three plug plates). Dosimeters were placed at the 0”0”0” (X,Y,Z), 4”0”0”, -4”0”0”, 4”0”2”, -4”0”2”, and 0”0”2” locations to measure the approximate dose of irradiation that each petri dish received. Dosimeters were used to gauge absorbed dose per petri dish and to ensure uniformity among treated petri dishes.

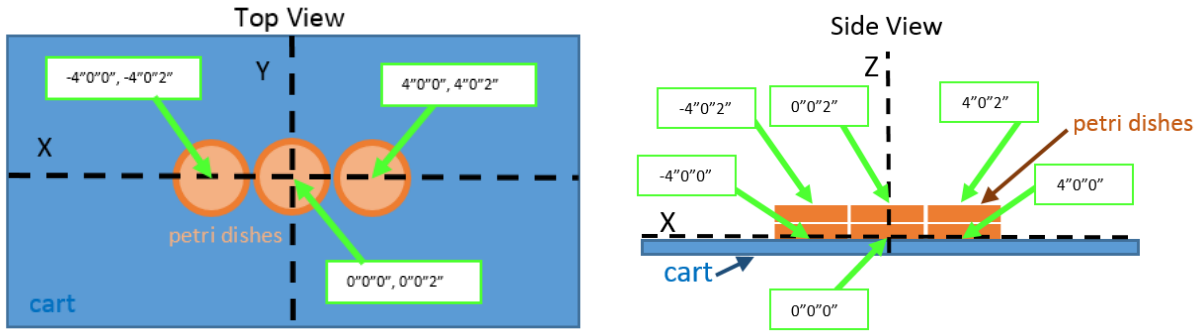

Figure 14. Alignment of petri dishes and dosimeters on irradiator cart

Four irradiation treatments were tested for *Stemphylium*: 0, 4, 6, and 10 kgry were tested. The dose served as a comparative because each treatment was tested separately due to the limitations of the irradiation equipment. For *Alternaria*, three treatment levels were tested: 0, 6 and 10 kgry.

Data were collected and analyzed using Microsoft Excel and SAS 9.3. In SAS 9.3 the Proc GLM function was used to perform the appropriate ANOVAs for each data set. Data were tested using Proc Univariate normal for normality and Proc GLM’s HOVTEST/Levene for homogeneity of variance. Additivity was not tested because there were more than one block/rep

combination for each data set. Proc GLM's means function /welch was used to analyze data sets that did not meet the homogeneity of variance assumption and for which no transformation was possible. All analyses were conducted with $\alpha = 0.05$. These experiments were conducted independently. Therefore, homogeneity of variance was established by using Levenes's test to be sure all of the 0 kgry controls performed the same between treatment dates.

To perform an ANOVA for *Stemphylium* spore germination across different treatment levels, the data needed to be transformed to meet the assumption of homogeneity of variance. A power transformation, with the germination data multiplied to the power of 0.34 was used to make the data fit the assumptions of the ANOVA.

Isolation and culture of pathogens:

Fungal pathogens were isolated from harvested (market maturity) Italian sweet basil. Fungal isolations were be made by cutting the tissue interface between healthy tissue and diseased lesions in approximately 1 cm sized cubes, surface sterilizing sections in a 10% NaOCl solution for about 5 seconds, and placing the sterilized tissue on water agar. Single hyphal tips were cut from the fungi that grew out and were grown on 10% V8 agar. Single spore cultures were obtained by glass needle transfers on new V8 agar plates and grown out as a pure stock cultures for 7 days. For *Stemphylium* spores did not grow out naturally so cultures were subjected to 24 hours of complete darkness to induce sporulation after six days of growth in light.

Effects of irradiation on *Stemphylium* and *Alternaria* spore germination:

Spore germination tests were conducted by collecting spores from pure cultures, and spreading them on 4 inch water agar plates. A sterilized glass rod with a rounded end was rubbed on the V8 cultures, and then smeared evenly onto the water agar with 3 petri dishes for treatment and three for controls. Control petri dishes were placed into a cardboard box and placed in the irradiation facility office which was similar in temperature to the conditions the petri dishes in the irradiator were exposed to. The plates were then irradiated and spore germination was determined per plate and compared with non-irradiated controls. Germinated spores were counted under a light microscope at 400X magnification. A germinated spore is defined as a spore producing a germ tube that is longer than the diameter of the spore. A second set of controls determined the percent of spores that germinated over the duration of time that it took for the treated controls to be irradiated. For instance, if a treatment required 2 hours to reach 4 kgry, a separate control was made to evaluate germination after 2 hours of being on water agar. This was to prevent false positives by subtracting the germinated spores from this control. Efficacy of treatments was determined by assessing how many spores germinated after the treatment.

Effects of irradiation on *Stemphylium* and *Alternaria* mycelial growth:

Mycelial growth was evaluated by using 7mm diameter plugs of fungal cultures from a mother plate. The mother plate was grown on 10% V8 agar under fluorescent light at 25°C for 7

days. The plugs were placed top side up onto the water agar. Three plugs were placed on each petri dish, with a total of six petri dishes inoculated in this manner. Three plates were used as controls and three were used for treatment. Each plug was individually accounted for. Colony size was measured at three locations with point of greatest growth, and averaged. Measurements were made with a ruler placed under the petri dishes and viewed with a dissecting microscope. Effective dose was calculated by assessing the amount of post treatment growth in millimeters from the plug.

IV. Results

Stemphylium:

Irradiated *Stemphylium* survived irradiation of up to 10 kgry. Average spore germination at 0 kgry, after 72 hours was 100% and average germination 72 hours after a 10 kgry dose was 1.43%. Gamma irradiation reduces germination of *Stemphylium* spores. A relationship between dose and spore germination exists. The same relationship exists with mycelial growth. At 0 kgry, fungal mycelial growth after 72 hours was 10.49 mm while mycelial growth 72 hours after a 10 kgry treatment was 0.333 mm. The trend lines of both spore germination and mycelial growth at different doses of irradiation rose over a period of 72 hours (Fig. 16, 18).

All of the 0 kgry were the same grouping and no significant heterogeneity of variance was found between the different treatment dates. With no difference at 0 kgry, different data were pooled and tested for homogeneity of variance. Regardless of hours post inoculation, dose of gamma irradiation has an effect on spore germination and mycelial growth (Fig. 15-18).

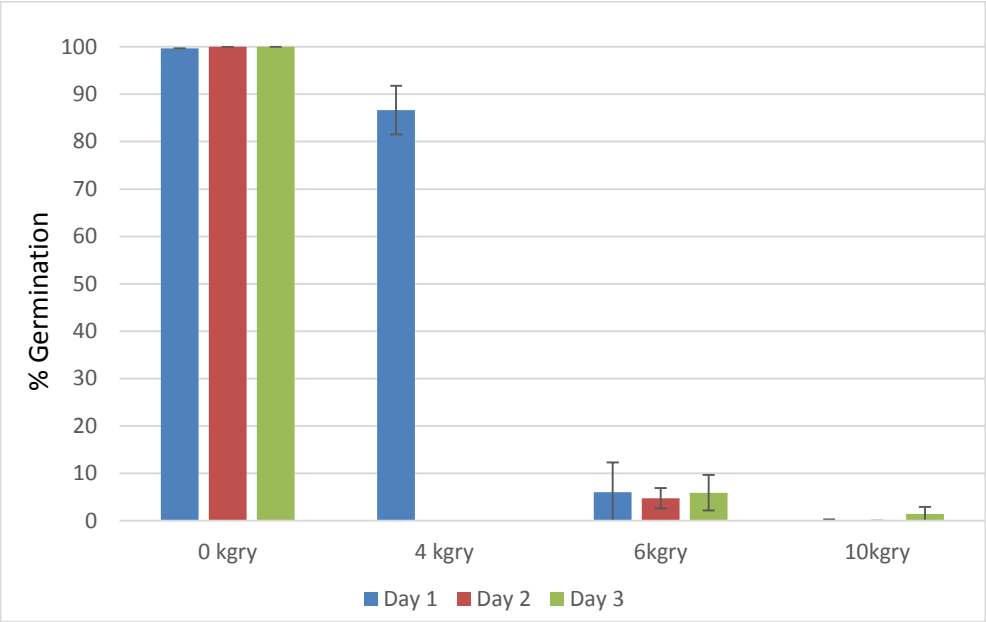

Figure 15. *Stemphylium* germination after exposure to different doses of gamma irradiation. Error bars are standard deviation.

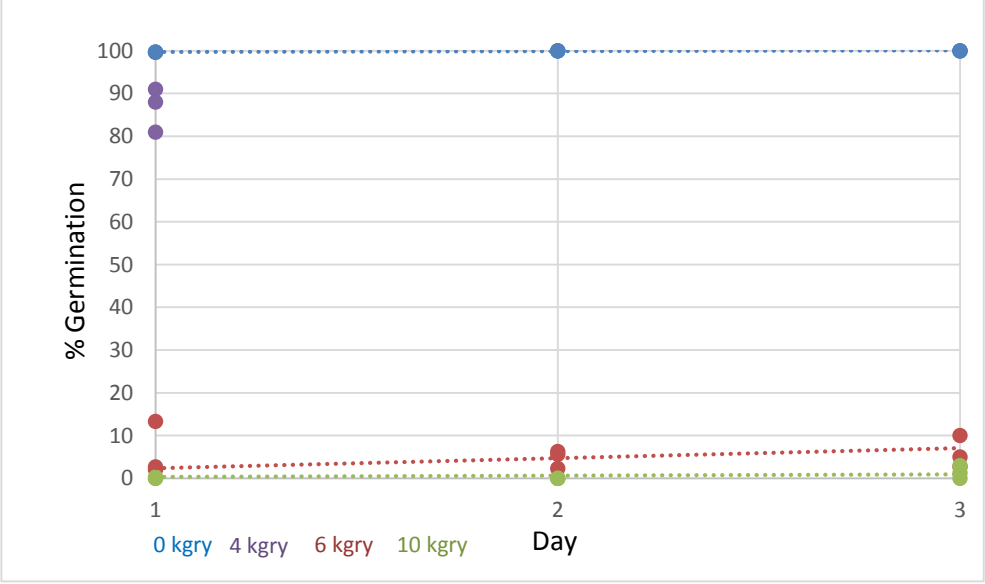

Figure 16. Scatter plot of *Stemphylium* germination after exposure to different doses of gamma irradiation.

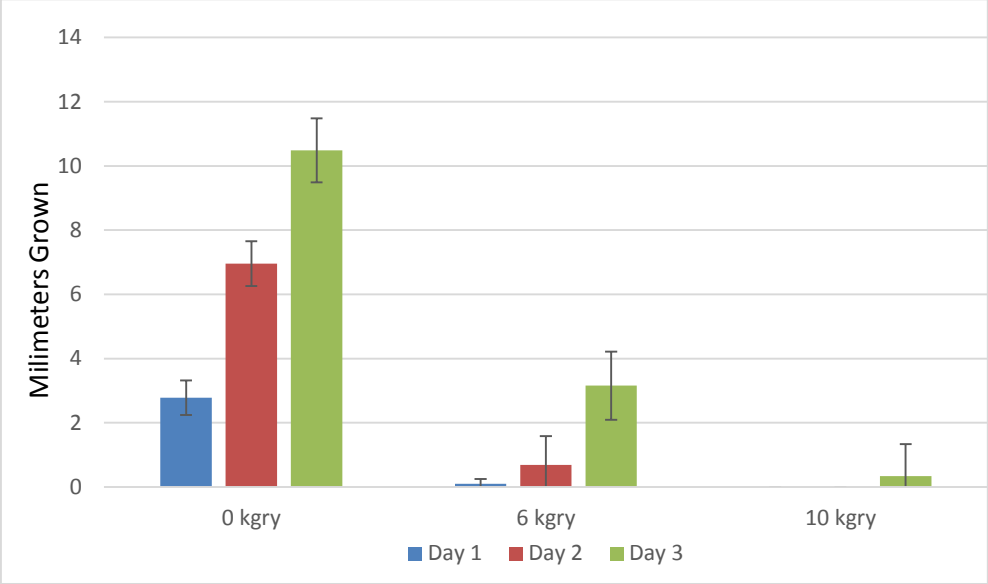

Figure 17. *Stemphylium* mycelial growth after exposure to different doses of gamma irradiation. Error bars are standard deviation.

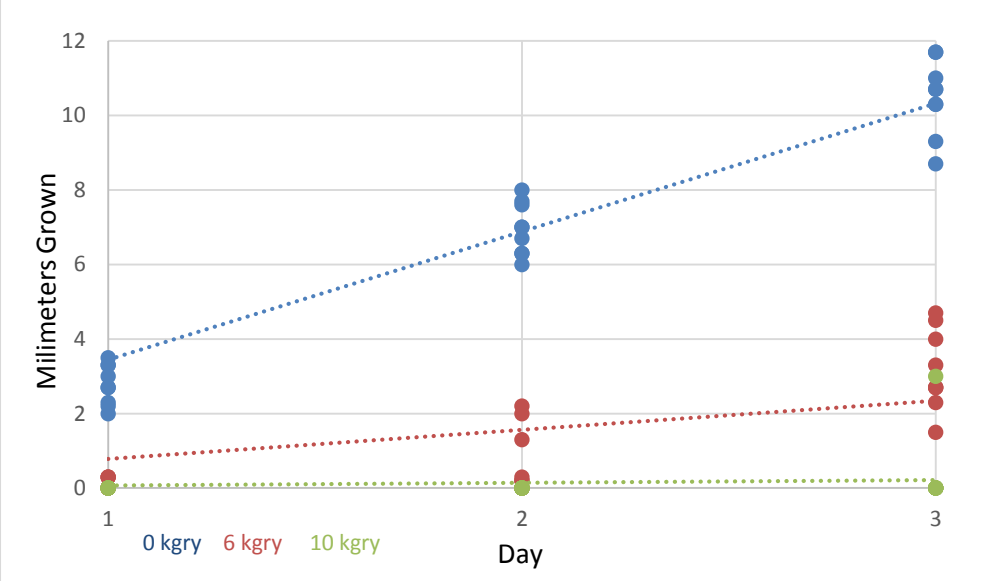

Figure 18. Scatter plot of *Stemphylium* mycelial growth after exposure to different doses of gamma irradiation.

Higher dose does lower the mycelial growth and spore germination of *Stemphylium* (Fig. 15-18). An ANOVA was preformed to determine if treatments were statistically significant. The regression analysis found a linear relationship within dose ($P<0.0001$) and a cubic relationship ($P<0.0001$) for germination with an R^2 value of 0.995 and a regression equation of $y=51.84x-18.84x^2+1.276x^3+99.9$. The data for mycelial growth were analyzed separately by day. The effects of dose were different on days 2 and 3 (P=0.0004 and 3 P=0.001). Day one was not different among doses (P=0.1809).

***Alternaria*:**

Alternaria survived at similar doses of irradiation to that of *Stemphylium*. At 0 kgry, germination averaged 98.1% after 72 hours whereas spores treated at 10 kgry averaged 9.9% germination after 72 hours. For mycelial growth the mean radial growth was 9.43mm at 0 kgry 0.667mm after 72 hours at 10 kgry (Fig. 19, 21).

Data were homogeneous and pooled for analysis. Because germination and mycelial growth did not meet the assumptions of the ANOVA, each day was analyzed separately using Welch's ANOVA. Dose was significant at day 1 for spore germination (P=0.0013) and mycelial growth ($P<0.0001$), at day 2 for spore germination ($P<0.0001$) and mycelial growth ($P<0.0001$), and at day 3 for germination ($P<0.0001$) and mycelial growth (P=0.0004) (Figs. 19-22).

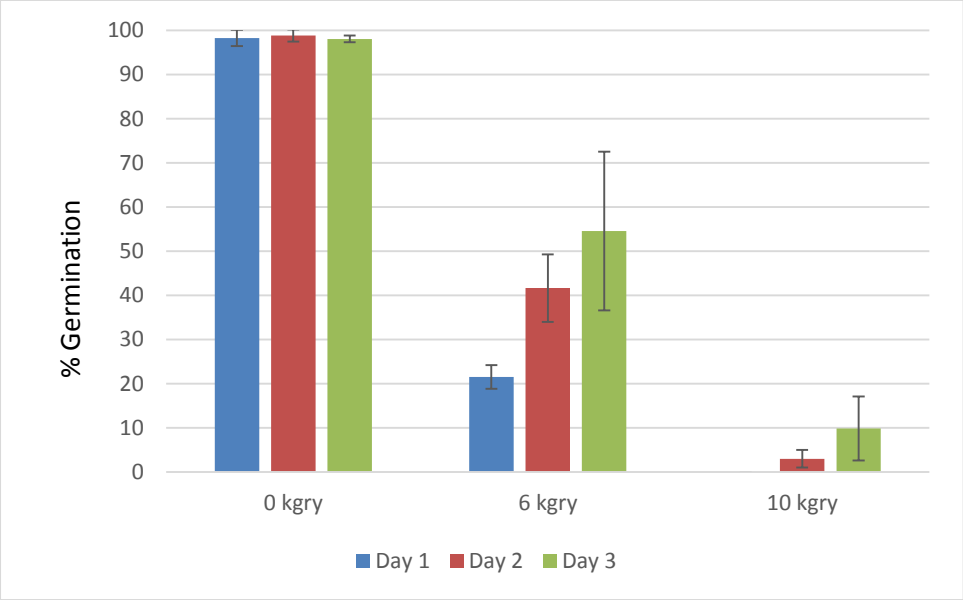

Figure 19. Responses of *Alternaria* spore germination exposed to different doses of gamma irradiation. Error bars are standard deviation.

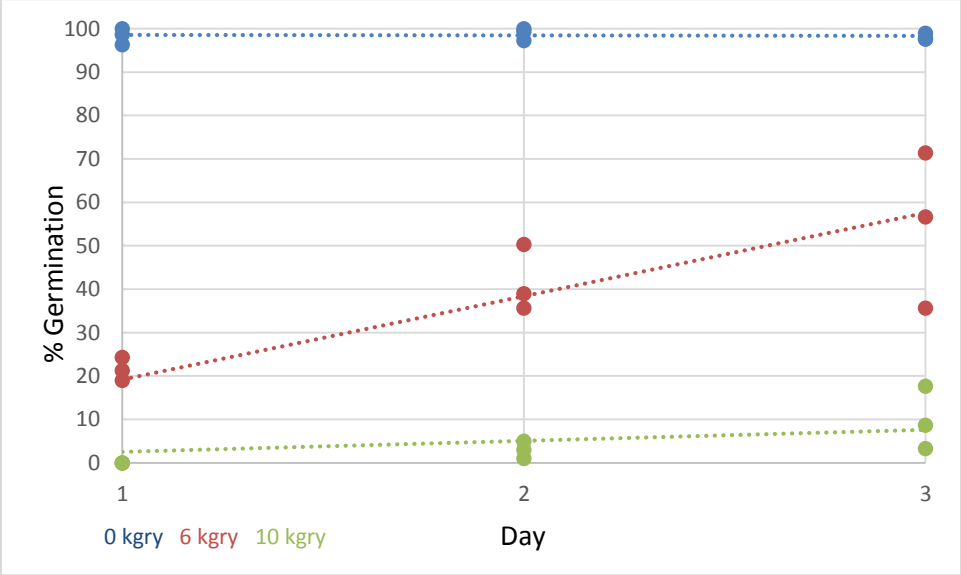

Figure 20. Scatter plot of responses of *Alternaria* spore germination exposed to different doses of gamma irradiation.

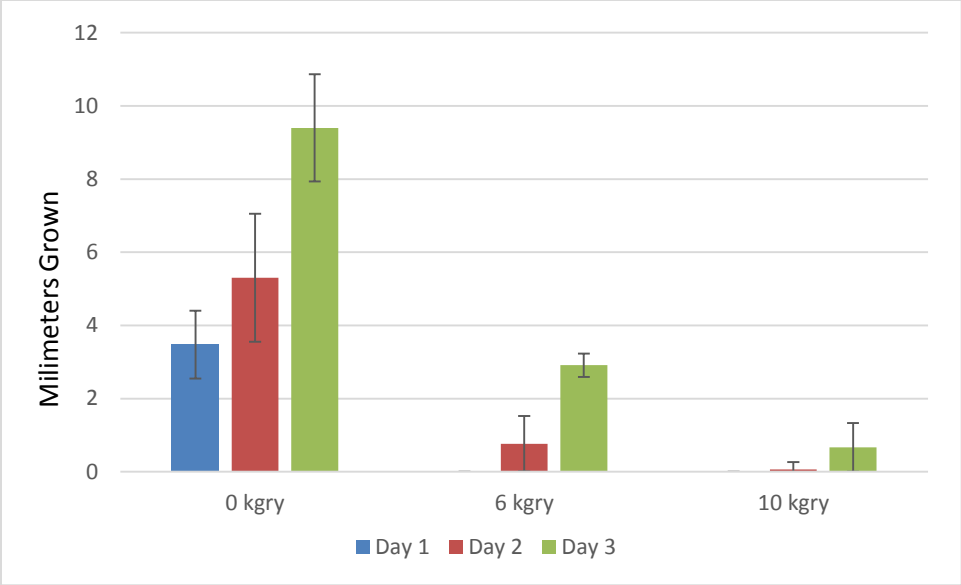

Figure 21. Responses of *Alternaria* mycelial growth exposed to different doses of gamma irradiation. Error bars are standard deviation.

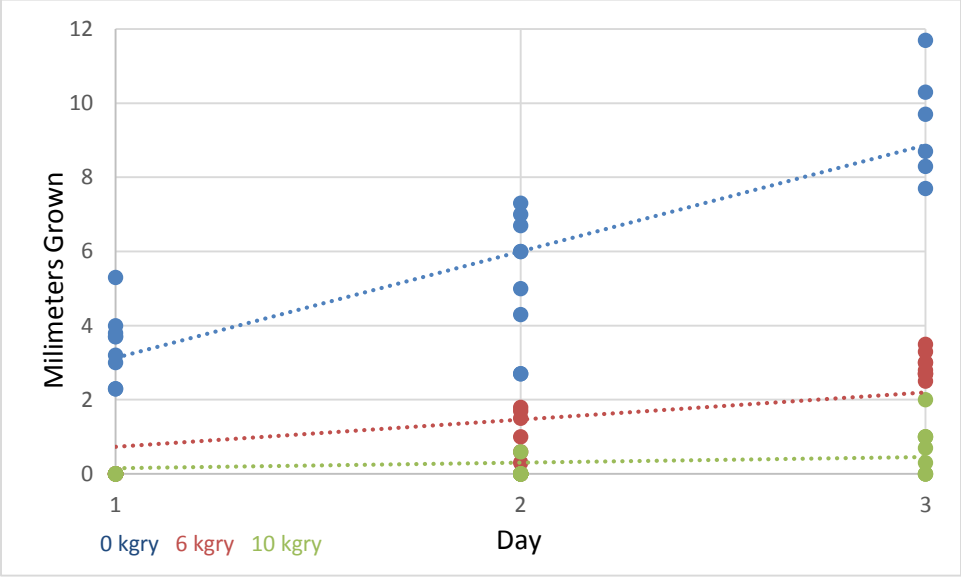

Figure 22. Scatter plot of responses of *Alternaria* mycelial growth exposed to different doses of gamma irradiation.

V. Discussion

Further tests at doses of 2 kgry and 8 kgry would give a much more accurate model for prediction of spore germination at any given dose of absorbed ionizing radiation. Unfortunately, an accurate model may not be formed here for *Alterneria*, as there are not enough doses tested to give a true understanding for a predictive model. This is especially evident because, in *Alternaria*, which performs similarly to *Stemphylium*, data were collected for only three doses, not four as in *Stemphylium*. The 4 kgry dose for *Stemphylium* gave insight as to how fungi perform when exposed to gamma irradiation, the effects of dose should have a significant cubic effect if *Alternaria* does indeed perform similarly to *Stemphylium*.

An accurate predictive model of *Stemphylium* and *Alternaria* spore germination and mycelial growth rates will require additional dose levels. Doses around 4 kgry and 7-8 kgry are appropriate to test if an accurate model for prediction is to be developed. The treatments of up to 10 kgry did not eliminate *Stemphylium* or *Alternaria*. Spores germinated and mycelial growth continued.

Dematiaceous fungi with melanized mycelia and conidia have a higher tolerance to radiation than other types of fungi (Calado et al., 2014). Many of these dematiaceous fungi, including *Alternaria* and *Stemphylium*, also have multicellular spores which also confer an advantage over single celled spores because spores can germinate from any one of their cells (Cavalcante et al., 1993). This makes the chance of survival for fungi like *Stemphylium* and *Alternaria* much higher when they are treated with irradiation. If one spore's cell's DNA is

irreparably damaged, other cells in the spore can ensure survival. In addition, dematiaceous fungi by definition are spores with darker color and tend to be those which are subjected to great amounts of ionizing radiation in their environments, mostly above ground and on leaves, flowers or fruit (Ellis, 1971). Concurrent studies being performed with *Phytophthora palmivora* (a relative of *Peronospora belbahrii*) elude to this. *Phytopthora palmivora* has clear/white colored sporangia and zoospores. This organism is disseminated by water, not air as with *Peronospora belbahrii* which has dark sporangia. At absorbed doses of 700 gry, *P. palmivora* had no spore germination and no mycelial growth for three days (Dragich et al., unpublished). At 400 gry, infection of papaya fruit was stopped, and *P. palmivora* was not recoverable (Dragich et al., unpublished). This is in contrast to *P. belbahrii* which had viable spores at absorbed doses of 2 kgry. These studies were done in a parallel, related research project.

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