

FUNGICIDE-INDUCED HORMETIC EFFECTS IN  
PLANT PATHOGENIC FUNGI AND OOMYCETES

By

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PLANT PATHOGENIC FUNGI AND OOMYCETES

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Abstract:

Fungi and Oomycetes pathogens are the causal agents of several plant diseases that often require chemical control. Fungicides are one of the main components in the management of such diseases. Intensive use of few fungicide active ingredients with the same mode of action without rotation result in selection of resistant isolates. Previous dose-response studies have reported increased growth, virulence and mycotoxin production in such resistant isolates when exposed to low doses of fungicides. Non-target effects of sublethal doses of fungicide, which can trigger beneficial responses in fungal and oomycete pathogens, can be attributed to “fungicide hormesis”. Although fungicide hormesis is often misinterpreted as experimental error and is overlooked, it is a highly reproducible phenomenon and can be well characterized by using proper experimental design and statistical tools. The objective of this study was to study the quantitative feature of fungicide hormesis. The first objective determined fungicide hormesis to be significant among resistant *Pythium* species: *P. irregulare* and *P. ultimum*. Two assays considering mycelial growth as endpoint were developed for this purpose. We found significant stimulation in growth area and total dry weight in both species at sublethal doses of mefenoxam. The second objective studied ubiquitous occurrence of fungicide hormesis among population of resistant isolates within *Sclerotinia homoeocarpa*. Twenty eight isolates of *S. homoeocarpa* were used to assess fungicide hormesis at subinhibitory doses of thiophanate-methyl. After multiple cycles of exposure to low doses of thiophanate-methyl, 17 isolates were determined to be thiophanate-methyl resistant, of which 81% showed statistically significant growth stimulation (2.8-19.7%). These observations reflected a high frequency of fungicide hormesis among resistant isolates of *S. homoeocarpa*. Overall, our results support the hypothesis that fungicide hormesis is common among fungal and oomycete plant pathogens. *In vivo* studies based on field and greenhouse trials are needed to assess the impact of fungicide hormesis on plant health. Nonetheless, it is recommended that chemical hormesis should be considered and properly assessed as a part of disease management strategies.

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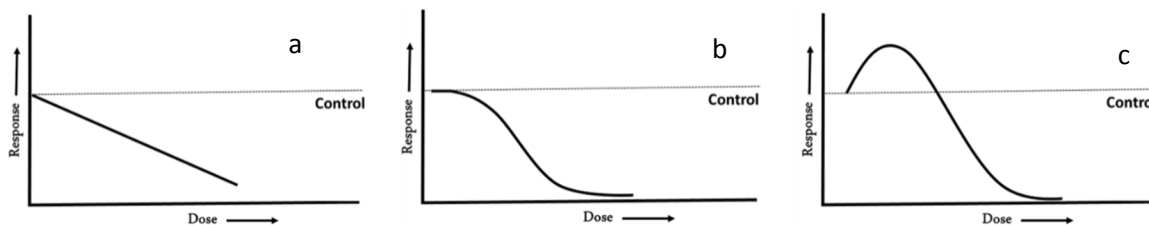
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## CHAPTER I

### INTRODUCTION

In toxicological and risk assessment studies, estimation of dose response relationship provides key information regarding safe levels of exposure to radiations and chemical inhibitors, also referred to as stressors. Dose-response can be characterized using three different models, the linear model, the threshold model, and the hormetic model. In the linear model that is often used to represent radiation dose-responses, responses decrease or increase with increasing doses (Figure 1a). In the threshold model, no response is expected until the dose of a stressor reaches a particular concentration (Figure 1b). Finally, the hormetic model is characterized by low dose stimulation and high dose inhibition (Figure 1c). The first two models follow monotonic patterns and the hormetic model follows a biphasic pattern, producing dose responses represented by inverted U or J shaped curves (Calabrese & Baldwin. 2003).



**Figure 1.** Dose-response curve outlining three basic models

(a) Linear model, (b) Threshold model, (c) Hormetic model

Hormesis is a toxicological phenomenon whereby biological systems exhibit opposed responses between high and low doses of a stressor, usually low dose stimulation and high dose inhibition (Calabrese & Baldwin. 2002). Hormetic responses have been described in the scientific literature as universal, independent of stressors, organism, or experimental design. Stimulatory responses are believed to occur either from direct stimulation (Szabadi. 1977, Calabrese. 2015) or from overcompensation to an initial disruption in homeostasis (Stebbing. 1981).

Biphasic dose-responses were overlooked in past toxicological and risk assessment studies as they were considered random fluctuations of responses; therefore data were often manipulated to fit monotonic models (Deng et al. 2000). During the last three decades, however, Edward Calabrese and a few other scientists made extensive efforts to demonstrate the prevalence of this phenomenon, and to understand the underlying origin (Calabrese & Baldwin. 1997, Calabrese & Baldwin. 2001, Stebbing. 1981, Davis & Svendsgaard. 1990). After extensive literature reviews, biphasic responses were proposed as ubiquitous in diverse biological systems including bacteria, fungi, Oomycetes, plants and animals (Branham. 1929, Calabrese & Howe. 1976, Gabliks et al. 1967, Hotchkiss. 1923, Jensen. 1907, Levy. 1998, Miller et al. 1945, Nickell. 1952, Southam & Ehrlich. 1943). Accumulation of extensive evidence of hormesis has issued new challenges to risk assessment criteria in different biological systems. In part because of these challenges, but mostly because of a prevalent lack of awareness among scientists and the general public about hormetic responses, this concept has been rarely studied, particularly in regards to plant pathogens.

Chemical control via pesticides is used in managing several plant diseases. However, repeated use of the same pesticides on pathogens over long periods of time, without rotating modes of action can result in the selection of resistant strains. Resistant strains tolerate therapeutic doses of pesticides, and in some oomycete and fungal species, resistant strains have been reported to have more vigorous growth and pathogenicity when exposed to sub-inhibitory doses of fungicides. Field or greenhouses crops can be exposed to low doses of fungicides through different routes including

the re-circulation of irrigation water after fungicide application, improper application, or subsequent dilution of the active ingredient due to environmental factors like sunlight and rainfall. The presence of hormetic responses, if ignored, can be detrimental to plant health and crop productivity as a result of increased growth rates of plant pathogens.

## **OBJECTIVES**

This thesis reports studies focused on two objectives that aim to improve the understanding of fungicide hormesis on plant pathogenic Oomycetes and fungi.

Previous studies reported hormetic effects on *P. aphanidermatum*, isolate P81, where low doses of mefenoxam stimulated radial growth of mycelium *in vitro* as well as increased the severity of damping-off of geranium seedlings (Garzon et al. 2011). Flores & Garzon (2012) found radial growth stimulation in *P. aphanidermatum* to be independent of the pesticides used, although hormesis was not observed in response to all the antifungal compounds tested (Flores & Garzon. 2012). Our first objective examined fungicide hormesis in two other *Pythium* species: *P. ultimum* and *P. irregulare* at low doses of mefenoxam. The experimental design was modified to evaluate hormetic effects on two mycelial endpoints: area and dry weight. Growth stimulation in each species was quantified and the variation in hormetic responses was determined. The second objective investigated the occurrence of hormetic responses in 28 isolates of *Sclerotinia homoeocarpa* collected from turfgrass in several US states. The sensitivity of each isolate to thiophanate-methyl was determined and dose-responses to sub-inhibitory doses of thiophanate-methyl on sensitive and resistant isolates were quantified and compared.

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## **CHAPTER II**

### **LITERATURE REVIEW**

#### **HISTORICAL FOUNDATION OF HORMESIS**

According to Henschler (2006), the first evidence of hormesis dated back to the 18<sup>th</sup> century when Rudolph Virchow studied the effect of potassium hydroxide and sodium on ciliae beating activity of ciliated epithelial muscle amputated from humans. He described biphasic dose responses characterized by increased beating of ciliae in response to low concentrations of the chemicals and complete arrest in activity at higher concentrations. In the 1880's, Hugo Schulz detected increased efficiency of fermentation by yeasts at low doses of multiple chemicals followed by inhibition at higher doses (Schulz. 1888). Later, in association with the homeopathic physician Rudolph Arndt, the unique biological responses was referred as the Arndt-Schulz law. However, the low-dose stimulation concept was undermined at the time particularly because they were not able to identify the mechanisms underlying such effect, and also because the stimulatory effects were associated with homeopathy, a widely criticized discipline, deemed a pseudoscience, put forward by Samuel Hahnemann in 1796. A few years later, Hueppe (1896) noticed similar effects in bacteria and formulated the "Hueppe rule" that addressed the limitations of the Arndt-Schulz law. Realizing the validity of Schulz's findings, Branham studied the sub-lethal dose effect of inhibitory chemical stressors like mercuric chloride, mercurochrome, metaphen, hexylresorcinol, chloramine-T, iodine, and sodium hypochlorite on CO<sub>2</sub> production by yeasts and reported outburst of gas production



during different phases of fermentation when exposed to sublethal doses of those chemicals (Branham. 1929). In 1943, Southam and Ehrlich observed growth stimulation in multiple strains of the wood decaying fungus, *Fomes officinalis*, when grown on media containing different concentration of extracts of red-cedar (Southam & Ehrlich. 1943). Based upon these findings, they were the first to use the term “hormesis”, derived from Greek word “*hormo = to excite*”, to describe their observations.

Currently, Edward Calabrese is considered one of the most influential scientist in this field. His interest toward hormesis began when he saw growth stimulation in *Mentha piperita* treated with low doses of phosfene, a growth retardant, in soil under experimental conditions (Calabrese & Howe. 1976). Eventually, Calabrese and collaborators investigated the occurrence of biphasic dose responses by reviewing past toxicological and risk assessment literature. Their preliminary evaluation found evidence of hormetic effects in 350 out of 585 studies. After thorough reevaluation of those 350 studies considering more stringent criteria, they reported that 82% of the 350 studies showed moderate to high evidences of hormesis (Calabrese & Baldwin. 1993, Calabrese & Baldwin. 1997a, Calabrese & Baldwin. 1997b). The extensive literature review by Calabrese and few other scientists like Stebbing revealed the occurrence of hormetic effects in diversity of organisms like bacteria (Calabrese & Baldwin. 1999, Hotchkiss. 1923, Miller et al. 1945, Morales-Fernandez et al. 2014, Woznica et al. 2013), Oomycetes (Fenn & Coffey. 1984, Garzon et al. 2008), fungi (Branham. 1929, Loefer et al. 1950, Loefer et al. 1952, Southam & Ehrlich. 1943), plants (Barcelo & Poschenrieder. 2002, Belz & Duke. 2014, Calabrese & Howe. 1976, Jensen. 1907, Mattson. 2008, Migliore et al. 2003, Migliore et al. 2000, Migliore et al. 2010, Nickell. 1952, Velini et al. 2008, Xin et al. 2013), arthropods (Guedes et al. 2010, Guedes & Cutler. 2014, Zuo et al. 2013) and animals (Gabliks et al. 1967, Hayes. 2007, Levy. 1998, Mattson et al. 2010). The inhibitory stressors included carcinogens (Elton et al. 1938), antibiotics (Campbell & Saslaw. 1949, Loefer et al. 1950, Loefer et al. 1952, Miller et al. 1945, Nickell. 1952), salts

(Hotchkiss. 1923, Jensen. 1907), metallic compounds (Stebbing. 2002, Stebbing. 1981,) and pesticides (Fenn & Coffey. 1984, Gabliks et al. 1967, Garzon et al. 2008), among others. The endpoints measured included growth parameters (Elton et al. 1938, Fenn & Coffey. 1984, Hotchkiss. 1923, Jensen. 1907, Miller et al. 1945, Nickell. 1952), CO<sub>2</sub> production (Branham. 1929), pathogenicity (Garzon et al. 2008) and several others. Based upon their findings, Calabrese has claimed that the progress in toxicological and other risk assessment areas might be delayed, and the field may suffer as a whole, if the renewed interest towards the concept of hormesis was ignored (Calabrese. 2002).

## **MECHANISTIC FOUNDATION OF HORMESIS**

Stebbing studied the effect of several metal and organometallic compounds on biomass growth of hydroid colonies of *Laomedea flexuosa* (Stebbing. 1982b) at different intervals of time. Based upon his findings and extensive review of several toxicological studies, he proposed that hormetic response might occur due to an initial overcompensation as a result of a disruption in homeostasis (Stebbing. 1981, Stebbing. 1982, Stebbing. 1998, Stebbing. 2003). According to Calabrese (Calabrese. 2015), the theory of overcompensation was first reported in the work of Townsend (1896), who investigated plant growth rates at four specific time intervals (1, 2, 3 and 8 days) following plant stress caused by multiple doses of ether. High doses resulted in a substantial reduction in growth over a 7 day period. In contrast, low doses resulted in initial 25% reduction in plant growth rate at day 1 followed by consistent stimulatory response (two fold increase over the control) at day 3. He stated that rapid recovery and stimulation was apparent in 6 to 24 hours after a small injury, which continues for one to several days. As for severe injury, there was a period of inhibition of growth rate, and the time for recovery was dependent upon degree of injury. The work of Townsend was carried on by Branham (Branham. 1929), Chavarria & Clark. (1924), Smith

(Smith. 1936, Smith. 1935) and Sperti et al. (1937) in fungi and their results supported the overcompensation hypothesis.

Elmer Szabadi (Szabadi. 1977, Calabrese. 2015) investigated several biphasic responses in past pharmacological literature that reported one stressor, particularly toxins, affecting two receptor subtypes. One subtype affected a stimulatory pathway at a lower concentration, while the other subtype affected an inhibitory pathway at a higher concentration. These findings supported a direct stimulatory response as an alternate possible hormetic mechanism. Calabrese (2013) thoroughly reviewed over 400 studies on receptor/signaling pathways to understand the mechanisms underlying hormesis and linked them to the ideas put forward by Stebbing and Szabadi. The review suggested that hormetic effects results from very diverse mechanisms. Although striking progress has been made in understanding the mechanisms underlying hormesis, none of the proposed mechanisms provides complete explanation of the experimental observations. Thus, hormesis remains a complex process comprising interplay of several factors.

## **HORMESIS IN PLANT PATHOGENIC OOMYCETES**

Fenn and Coffey mentioned growth stimulation at low doses of  $H_3PO_3$  for *Pythium ultimum* and *Pythium myriotylum* (Fenn & Coffey. 1984). Likewise, *Phytophthora undulata* were reported to be stimulated by low doses of hymexazol (Kato et al. 1990). Additional evidence of growth stimulation was demonstrated by Zhang et al. (1997) when he examined *in vitro* growth stimulation in *Phytophthora infestans* by metalaxyl, a commonly used fungicide to control Oomycetes. They found that three out of four metalaxyl resistant isolates had vigorous growth stimulation with increased biomass of aerial hyphae when grown on a media amended with 20  $\mu$ l/ml of fungicide. They also observed that one of the three isolates grew more in media containing metalaxyl and less nutrient. Based on these observations, they inferred metalaxyl to be beneficial to resistant isolates

of *Phytophthora infestans* under low nutrition. Moorman and Kim (2004) observed increased growth of mycelium in some strains of *Pythium aphanidermatum*, *P. irregulare* and *P. ultimum* that were resistant to both propamocarb and mefenoxam. They reported that isolates of the three species were stimulated by propamocarb at a concentration of 1 µg/ml; additionally a resistant isolate of *P. aphanidermatum* was stimulated by concentration of 1000 µg/ml (Moorman & Kim, 2004). Garzon et al. (2011) studied the effects of low doses of mefenoxam on radial growth of mycelium and on plant disease severity. Mefenoxam resistant isolates of *Pythium aphanidermatum* and *P. cryptoirregulare* were grown in media amended with fungicides and an *in planta* assay was designed to record damping-off of young geranium seedlings at sub-lethal dose of mefenoxam. They observed up to 10% average stimulation of radial growth of mycelium and significant increase in damping-off of geranium seedlings up to 61 %. In an attempt to estimate hormetic effect with accuracy, Flores & Garzon. (2012) reported standardized protocols *in vitro* for detection and assessment of such effect using radial growth as endpoint. While validating the protocol, they examined growth of *P. aphanidermatum*, *Rhizoctonia solani* and *R. zae* exposed to multiple doses of ethanol and in the case of *P. aphanidermatum*, the fungicides propamocarb and cyazofamid. All the species showed growth stimulation at low concentration of ethanol while *P. aphanidermatum* showed growth stimulation to both fungicides tested.

## **HORMESIS IN PLANT PATHOGENIC FUNGI**

The first evidence of hormesis on a fungal pathogen was reported by Southam and Ehrlich (1943), who demonstrated growth stimulation in *Fomes officinalis* cultured *in vitro* at low doses of red-cedar heartwood extract. In an experiment conducted by Hessayon (1951) to determine the production of trichothecium, an antifungal compound produced by *Trichothecium roseum*, on different soil types, he found that sublethal doses of trichothecin induced mycelial growth in

*Fusarium oxysporum*. Baraldi et al. (2003) studied 41 thiabendazole (TBZ) resistant isolates of *Penicillium expansum* and observed improved germination in seven isolates when compared to the control. They stated that such stimulation in germination rate might have resulted from the ability of resistant isolates to metabolize the fungicide as a nutrient compound. Audenaert et al. (2010) studied the effects of subinhibitory doses of the fungicide prothioconazole on mycotoxin deoxynivalenol (DON) production *in vitro* and *in planta* by *Fusarium graminearum*. Their results suggested that small doses of prothioconazole led to higher production of the H<sub>2</sub>O<sub>2</sub> which in turn triggers DON production. Landry et al. (2011) evaluated the effects of multiple fungicides on sphagnum grayling disease caused by the fungus *Lyophyllum palustre*. They found substantial increase in radial growth when the fungus was grown in media amended with low concentrations of propamocarb compared to non-fungicide amended media. Flores & Garzon (2012) reported radial growth stimulation in *Rhizoctonia* spp. at subtoxic doses of ethanol. Recently, Zhou et al. (2014) observed hormetic effects of dimethachlon fungicide on different isolates of *Sclerotinia sclerotiorum*. Eighteen out of 58 isolates had increased growth rates compared to the non-treated control when grown on media amended with 0.5 - 4 µg/ml dimethachlon. They also found increased virulence on detached leaves of oilseed rape after spraying plants with dimethachlon at a concentration of 2 µg/ml.

Evidence of hormetic responses in fungi have also been reported in response to radiation. Smith (Chavarria & Clark. 1924, Smith. 1936, Smith. 1935) reported radiation induced hormetic effects on *Fusarium*. Her experiment was based on dose-time responses where she exposed *Fusarium* to nine doses of radiation (0.05-15 min) and observed the effect on mycelial growth. At 24 hours, all doses showed inhibitory response; however, at 72h all doses except the highest dose produced stimulatory response of 15-40% compared to the control.

There are several other mentions of pesticide-induced hormetic effect in the phytopathology literature (Hocart et al. 1990, Parra & Ristaino. 2001). However, these effects were

not properly highlighted either because such effect was overlooked as experimental error or due to the lack of awareness about chemical hormesis among the phytopathological community.

## **QUANTITATIVE EVALUATION OF HORMETIC EFFECT**

Guidelines regarding evaluation of hormetic effects based upon quantitative approach was first mentioned by Calabrese & Baldwin (1997b). The guidelines recommended fulfilment of three different criteria i.e. 1) determination of no observable adverse effect level (NOAEL); 2) assessment of the effect of five equally spaced doses below NOAEL to provide enough data for detection; 3) separation between experimental doses below the NOAEL should be less than one order of magnitude, since the hormetic zone is expected within a ten-fold range. Assessment of data is crucial when demonstrating hormetic effects. The criteria for evaluating hormetic effects include 1) strength of evidence; 2) soundness of data; 3) consistency, and 4) biological plausibility (Crump. 2001). Hormetic responses are often difficult to detect because of background noise due to initial fluctuation in responses. However, hormetic responses are highly reproducible and can be easily differentiated from background fluctuations by using appropriate experimental designs and statistical analysis tools (Calabrese & Baldwin. 1997b).

Several methods, including parametric (Schabenberger et al. 1999) and non-parametric (Deng et al. 2000) approaches have been proposed to accurately estimate hormetic responses. The log-logistic model is commonly used approach for dose response studies and expresses mean dose responses as a sigmoidal, monotonic curve that has increasing or decreasing function in log (dose) (Schabenberger et al. 1999). However, the log logistic function is not able to estimate biphasic responses such that Brain and Cousens (1989) proposed a modified log-logistic model to better fit biphasic responses by introducing a parameter ( $\gamma$ ) that measures initial rate of increase at small dose. However, Brain and Cousens model is limited in their application, since it will not produce a

curve when the slope is less than 1 (Cedergreen et al. 2005). Schabenberger et al. (1999) and Vanewijk & Hoekstra (1993) made further modifications to the Brain and Cousens model that allow estimation of several hormetic dose parameters like EC<sub>50</sub>, NOAEL, and the maximum stimulation dose (MSD) that are highly relevant to predict the nature of pathogens. Flores & Garzon (2012) used a Brain Cousens model based approach modified by Schabenberger to detect hormetic effect in fungal pathogens. Estimation of parameters and curve modeling was done using coding provided in the literature to perform analysis in SAS (Schabenberger et al. 1999).

## **BIOLOGY AND MANAGEMENT OF *PYTHIUM* SPECIES**

*Pythium irregulare*, Buisman, (1927) and *P. ultimum*, Trow, (1901) are currently classified under kingdom Straminipila together with the heterokont golden-brown algae (Baldauf et al. 2000). These fungus-like organisms belong to the phylum Oomycota, class Oomycetes, order Pythiales, family Pythiaceae and genus *Pythium*. The genus *Pythium* comprises about 140 recognized species (Bala et al. 2010, Levesque. 2011, Vanderplaatsniterink. 1981) and are taxonomically unrelated to fungi. Although the genus *Pythium* is under revision, the genera into which its species will be distributed are still under discussion (Abad et al. 1994).

Most *Pythium* species reproduce both sexually and asexually. Sexual reproduction takes place by fusion of a male gametangium (antheridium) with a female gametangium (oogonium), each contributing a haploid nucleus, producing very resilient diploid structures called oospores that serve as resting structures. Oospores can survive in soils for several year and germinate to produce coenocytic mycelia. Asexual reproduction takes place by formation of structures called sporangia where zoospores are formed, matured and released. Zoospores are single celled diploid motile propagules that can swim freely in water due to the presence of two structurally distinct flagella. The anterior flagellum, also known as tinsel flagellum, is a brush like structure that may have a

sensorial function, while the posterior flagellum, known as whiplash flagellum, is responsible for most of the mobility of zoospore (Agrios. 2005). Sexual reproduction occurs almost exclusively by self-fertilization such that most strains of both *P. irregulare* and *P. ultimum* are homothallic for many loci. Previously, all *Pythium* species were considered as universally homothallic until the 1940's when extensive search for mating type based on sterol- induced mating reaction revealed numerous evidences of heterothallism (Hendrix & Campbell. 1973).

*Pythium* are ubiquitous in nature and found in different ecological niches in the form of saprotrophs, facultative plant pathogens and sometimes as mycoparasites of some phytopathogenic fungi and other oomycetes (Horner et al. 2012). They are called water molds as they prefer high humidity and environments with free water. Infection of plant is favored by high humidity. Infested irrigation sources allow dissemination of the pathogen through zoospores. Root colonization and infection are favored by high moisture content and 50% saturation capacity of soil respectively (Hendrix & Campbell. 1973). Unlike many *Pythium* species that are pathogenic at high temperature, *P. ultimum* and *P. irregulare* prefer comparatively lower temperatures (Hendrix & Campbell. 1973).

*Pythium* strains survive in soil either as saprotrophs or as oospores. Saprotrophic mode of survival is rare and is favored by soil moisture and limited or no competition for nutrients in the soil (Griffin. 1963). The oospore is the predominant structure for long term survival. Competition for nutrients with other microorganisms or the lack of nutrients results in reduction and eventual arrest of mycelial growth, ultimately inducing formation of propagules and resting structures, including zoospores and sporangia (for short period of time) and thick-walled oospores (for longer periods) (Agrios. 2005, Stanghellini & Hancock. 1971). *P. ultimum* oospores are highly resilient and are able to survive under harsh environmental conditions. They can survive at -18°C for up to 2 years and in air dried soil for over a year (Hoppe. 1996, Munnecke & Moore. 1969). Oospores



can survive *in vitro* storage in water agar in sterile water for over five years or more (Abad et al. 1994).

Chemical stimuli released by host seeds or root exudates attracts zoospores and oospores towards host tissue. Infection starts once the encysted zoospores and/or oospores attach to the host tissue and then forms a germination tube that penetrates the host tissue directly (Agrios. 2005). Infection can also take place indirectly by production of sporangia by oospores.

*Pythium* are known to cause disease in a wide variety of economically important plants, ranging from ornamental crops produced in greenhouses and nurseries, to food crops and turfgrass in the field. Both *P. irregulare* and *P. ultimum* have wide host ranges infecting over 200 species of plants including fruits, cereals, grasses and many floricultural crops, such as geraniums, to name a few (Vanderplaatsniterink. 1981). The two species have been reported in almost every crop grown in greenhouses and nurseries (Moorman. 2015). *Pythium* targets juvenile tissues and confine their parasitism to seedlings, root tips and moist tissues of fruits and stems (Hendrix & Campbell. 1973). *Pythium* spp. cause pre- and post-damping-off of seedlings, crown and stem lesions, fruit rot, root rot, cottony blight and turf blight (Hendrix & Campbell. 1973, Kerns. 2008, Sanders. 1984). Pre-emergence damping-off results from infection in seedlings, prior to germination or shortly after germination, before they reach the soil surface. The infected seedlings turn brown and soft, followed by decomposition due to enzymatic activity of the pathogen and secondary infection by saprophytic bacteria and fungi. Post-emergence damping off takes place when seedlings rise from the soil surface after germination, become infected and turn water-soaked, and eventually collapse. Stem rot occurs in juvenile plants where water soaked lesions appear at the stem immediately above soil surface that eventually enlarge and kill the plant. Root rot occurs at almost every stage of plant development. The pathogen attacks root tips and form brown lesions followed by chlorosis and/or wilting. Symptoms include water soaked, brown roots with necrotic tips resulting in loss of vigor, chlorosis, stunting or complete plant collapse. *Pythium* blight starts with small brown spots on the

leaf surface that turns dark green color. The infected area becomes water soaked and mycelial growth is observed once the pathogen reaches turf blades. The mycelium that grows on foliar canopy of turf serves as inoculum and is able to penetrate leaves directly (Agrios. 2005).

Introduction of resistant cultivar and crop rotation practices are the most commonly used control measures to address pathogen induced plant diseases. However, such methods are not effective in controlling *P. irregulare* and *P. ultimum* due to the wide host range and limited number of resistant cultivars (Daughtrey & Benson. 2005, Koike et al. 2000, Martin & Loper. 1999). Management based on the integrated use of cultural, biological and chemical methods should be considered to prevent crop losses. Pythium diseases are hard to manage once there is infestation. Disease can be prevented to some extent by following common cultural practices like site selection with no prior known history of pathogenic *Pythium* infestation, use of certified seeds, irrigation management to avoid excessive soil moisture, proper aeration and changing the ratio of fertilizer and nutrients. Biological management include introduction of antagonistic organisms to maintain pathogenic populations to a point where there is no economically significant crop loss. Fungal species like *Streptomyces griseoviridis*, *Gliocladium catenulatum*, and *Trichoderma harzianum*, and bacterial species, like non-pathogenic strains of *Burkholderi cepacia*, are some of the commercially sold microorganisms used for biological control of Pythium diseases (Martin & Loper. 1999).

Chemical control has been widely used to control Pythium diseases. Limited number of pesticides are currently available to control *Pythium* species. Some of the most common fungicides for *Pythium* management include propamocarb, etridiazole, fosetyl-aluminum, metalaxyl and its R-enantiomer mefenoxam, azoxystrobin, pyraclostrobin, and cyazofamid. Metalaxyl is a phenylalanine compound and it is a racemic mixture of two enantiomers. Metalaxyl is a systemic compound that inhibits protein synthesis in Oomycetes (Monkiedje & Spiteller. 2005). This active ingredient was first registered in the U.S. in 1979 for use on cotton, potatoes, and tobacco. When

only R-metalaxyl is present it is known as mefenoxam. Mefenoxam is a predominant fungicide used to control *Pythium* and *Phytophthora* diseases. Repeated use of the same fungicide over a long period of time resulted in development of resistance that was first reported in turf (Sanders, 1984). At present, mefenoxam resistance has been reported in several states in the United States. Moorman et al. (2002) isolated *Pythium* from many infected plants in Pennsylvania and demonstrated that 32.5% of 120 isolates were mefenoxam insensitive. Simultaneous resistance to mefenoxam and propamocarb has also been reported (Moorman and Kim, 2004).

### **BIOLOGY AND MANAGEMENT OF *SCLEROTINIA HOMOEOCARPA***

Dollar spot was first reported in turfgrass by Monteith and Dahl (1932) in the United States and Canada. Initially, the causal agent for dollar spot was identified to be *Rhizoctonia* species (Monteith & Dahl, 1932), due to similarity in disease symptoms. In 1937, Bennett formally described the fungus *Sclerotinia homoeocarpa* as the causal agent of dollar spot disease. Unlike other *Sclerotinia* species that produces tuberoid sclerotia, *S. homoeocarpa* forms dark flake-like stroma that produces ascocarp upon germination (Bennett, 1937). Bennett obtained three strains of *S. homoeocarpa* from Australia, Britain and United States. Only few strains from British origin produced ascospores and were categorized as “perfect strains” (producing ascospores and conidia) and “ascigenous strains” (producing ascospores and microconidia). Isolates that did not produce ascospores were considered as non-spore-forming strains. Despite differences among the three strains, all were considered as a single species. Currently, they are placed in the kingdom fungi, division Ascomycota, class Ascomycetes, order Helotiales, family Sclerotiniaceae, and genus *Sclerotinia*. Most scientists believe that this fungus needs to be reclassified, however, due to complexity in understanding its reproductive structure, the classification remains controversial.

Based on the sequences of the ribosomal DNA, it is possible that it will be assigned to either of the genera *Lanzia*, *Moellerodiscus*, or *Rutstroemia* (Allen et al. 2005).

*S. homoeocarpa* affects more than 40 plant hosts most of which belong to the family Poaceae. Almost all varieties of turfgrass in United States are susceptible to dollar spot. Some cultivars of turfgrass are more resistant to dollar spot than other cultivars (Schroeder. 1996). *S. homoeocarpa* produces stromata that act as overwintering structures. These darkly pigmented structures are produced and remain on the margin of dollar spot lesions (Couch. 1995, Smiley et al. 1992)

The pathogen survives as dormant mycelium in infected plants or as a stromata (darkly pigmented survival structure and under favorable conditions, the mycelium grows and enters host tissues via injuries at the tip of the leaves, through stomata or by direct penetration (Fenstermacher. 1980, Monteith & Dahl. 1932, Endo. 1966). Baldwin & Newell (1992) reported fertile apothecia in turf and suggested that ascospores might play an important role as primary inoculum in the spring. However, the production of spores is very rare such that the primary source of fungal dissemination is limited to contaminated tools, water, wind, leaf debris and movement of humans or animals (Smith. 1955). Environmental factors contribute greatly to pathogenicity of the fungus. The optimal temperature for growth of *S. homoeocarpa* was reported as 26.8°C; however, it is able to survive from 4.5 to 32°C on PDA media (Endo. 1963). Peak disease occurs from late spring through autumn, at temperature ranges from 21 to 27°C and humidity higher than 85% (Couch. 1995, Endo. 1963, Smiley et al. 1992).

Disease symptoms start on individual leaf blades forming chlorotic lesions, followed by a water soaked appearance that later bleaches into straw-colored with tan to reddish-brown margin (Smiley et al. 1992). As the disease progresses, the lesion extends throughout the leaf and can take the shape of an hourglass (Smiley et al. 1992). The fungus then initiates infection to neighboring

leaf blades and affected area appear as circular, straw-colored patches in closely mowed turf. These unsightly patches initially take the size of a silver dollar, but later may unite into large, irregularly shaped spots on turf surface (Smiley et al. 1992). Symptoms of dollar spot varies with different host species and mowing height. Cob-web like mycelial growth of the fungus may also appear under a combination of warm weather (18-27°C) and morning dew (Monteith & Dahl. 1932).

Cultural control involves practices that reduce the environmental conditions unfavorable for the pathogen. Reducing the amount and duration of leaf/soil wetness, and maintaining appropriate soil nitrogen levels are some of the key cultural control measures. Since *S. homoeocarpa* requires free water on the surface of the leaf to infect turf, leaf wetness can be reduced by mowing and irrigating turf in early morning and pruning nearby trees and shrubs to increase air circulation and solar radiation (Williams et al. 1996). Irrigation to maintain soil moisture content above 75% of field capacity will reduce disease severity (Couch. 1995). Maintaining adequate nitrogen fertility supports plant health, minimizes damage and facilitates recovery from dollar spot disease. A lack of sufficient nitrogen (N) will predispose plants to infection and will increase disease severity (Endo. 1966). Although the pathogen requires N for growth and appressorium formation, plants that are N-stressed are more vulnerable to disease (Endo. 1966). Nonetheless, over-fertilization with nitrogen may cause an increase in dollar spot severity, and also will promote other diseases such as Pythium blight and brown patch.

Grasses, especially bentgrass used on golf greens, vary greatly in susceptibility to dollar spot. Cultivars such as L-93, A-1, Providence and Pennlinks have shown significant tolerance to dollar spot (Hurley. 1999). Recently, transgenic approaches have also been used to obtain resistance to dollar spot. Guo et al. (2003) successfully generated transgenic creeping bentgrass (*Agrostis palustris*) that showed delayed development of dollar spot. They generated transgenic creeping bentgrass plants expressing PR5K from *Arabidopsis thaliana*. PR5K is a receptor protein kinase whose extracellular domain is homologous to the PR5 family of pathogenesis-related proteins. The

PR5 proteins have been found to have antifungal activity by disrupting fungal plasma membranes. In a field test of plants inoculated with *S. homoeocarpa*, four of the eight transgenic lines showed delayed disease expression of 29 to 45 days, relative to the non-transgenic control plants. Extensive research has been conducted into the biological control of dollar spot that is broadly categorized into two strategies. The first strategy involves increasing the naturally occurring population of antagonistic microorganism by application of organic fertilizers, composts and sludge (Hoyland & Landschoot. 1993, Landschoot & McNitt. 1997, Liu et al. 1995, Nelson. 1991a). The second strategy involves use of non-pathogenic bacteria and other fungi to suppress disease (Nelson. 1991b, Schumann & Reuter. 1993, Walsh et al. 1999). Recently, disease forecasting by use of prediction models is being employed. Mills et al. (1982) and Hall & Rothwell (1984) proposed a model that required fungicide application with response to data based on weather condition, infection and symptoms development. Although these models easily measured variables to predict disease, none of them accounted for leaf wetness caused by dew and guttation. Walsh et al. suggested that although humidity and rainfall were factors in dew formation, actual leaf wetness duration was a more accurate indicator of available free water (Walsh et al. 1999).

Despite the use of other control measures, chemical control via fungicides has proved to be the most effective means of controlling dollar spot over the last 4 decades. Although *Pythium* blight is considered as the most critical disease in golf courses, Vargas (2005) found that large amount of fungicides are used to control dollar spot making it the most economically important turf disease (Vargas. 2005). Some programs require application of fungicides as frequently as once every 7-14 days (Latin. 2008). Frequent application of fungicides throughout a growing season has resulted in selection of fungicide resistant fungal strains, which is a challenge for the turfgrass industry. Some of the commercially available fungicides to control dollar spot include benzimidazoles, demethylation inhibitors, dicarboximides, chlorothalonil, anilazine and heavy metal based fungicides (Latin. 2008, Settle et al. 2001). Resistance have been reported in each of

these groups of fungicides (Bishop et al. 2008, Cole et al. 1968, Cole et al. 1974, Detweiler et al. 1983, Goldberg & Cole. 1973, Golembiewski et al. 1995, Hsiang et al. 2007, Hsiang et al. 1997, Jo et al. 2004, Massie et al. 1968, Nicholson et al. 1971, Warren et al. 1974). The problem is worse as strains of *S. homoeocarpa* that are resistant to one fungicide are often cross resistant to other fungicides that have the same mode of action (Cole et al. 1974, Golembiewski et al. 1995, Warren et al. 1974). The Benzimidazole group of fungicides, such as thiophanate-methyl, are systemic fungicides introduced in the late 1960s. Benzimidazole is a heterocyclic aromatic organic compound that interferes with the nuclear division, particularly by inhibiting DNA synthesis (Yang et al. 2011). Although this group of fungicides were very effective early on even at low doses, pathogens quickly acquired resistance (Fenstermacher. 1980, Smith et al. 1989, Vargas. 1994) and these resistant strains were found to cause more severe disease in creeping bentgrass (Vargas. 1994). Pathogens with resistance to a single group of fungicides were also reported to influence the same isolate to develop resistance to other fungicide groups; however, isolates that were multi-resistant were not persistent in turf after discontinuation of that group of fungicides (Vargas. 1994, Vargas et al. 1992).

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## CHAPTER III

### SUBINHIBITORY DOSE OF MEFENOXAM INDUCE HORMETIC EFFECT IN *PYTHIUM* SPECIES

#### ABSTRACT

*Pythium* species cause many plant diseases including blight of turfgrasses, damping-off, and root rots. Isolates develop fungicide resistance from the repeated use of single active ingredient without rotation making disease management difficult. Resistant isolates of few soilborne plant pathogenic oomycetes and fungi have been reported to have stimulatory responses when exposed to low doses of fungicides. Such effect can be explained by the toxicological phenomenon called hormesis. Previous studies have demonstrated increased radial growth and pathogenicity in a mefenoxam resistant isolate of *P. aphanidermatum* by sublethal doses of mefenoxam. This study aimed to examine hormesis in mefenoxam-resistant isolates of two *Pythium* species, *P. ultimum* and *P. irregulare*, and the reproducibility of results in two different growth related endpoints: total growth area and total dry mass weight. Two assays were conducted to determine stimulation in growth area and dry weight of mycelium using ten concentrations of mefenoxam ranging from 0.01 to 1,000 µg/ml. *P. ultimum* showed growth stimulation of 8.61 and 11.95% in growth area and in dry weight respectively. *P. irregulare* displayed 12.6% increased growth stimulation in mycelial growth and 14.09% increase in dry weight. Both assays were able to detect statistically significant hormetic effects in each species with no significant differences between endpoints.

Keywords: fungicide hormesis, oomycetes, fungal, low-dose, growth stimulation, mefenoxam

## INTRODUCTION

Hormesis is a toxicological phenomenon characterized by two types of responses to stressors: stimulation at low doses and inhibition at higher doses (Calabrese & Baldwin. 2002). The mechanisms involved in such response are not well understood. However, evidence suggests that hormesis might result from direct stimulation (Calabrese. 2015) or initial overcorrection due to disruption in homeostasis (Stebbing. 1982). In 1943, Southam and Ehrlich observed multiple strains of wood decaying fungus, *Fomes officinalis*, displaying low dose stimulation and high dose inhibition to red cedar extracts and introduced the term “hormesis” to describe the stimulatory effect they observed. Extensive literature reviews by Calabrese and a few other scientists have revealed that hormetic effects were triggered by a wide range of stressors, including inhibitory chemicals and radiation, using different endpoints, such as growth, longevity, mycotoxin level, among others, and is not only limited to oomycete and fungal organisms but is also present in bacteria (Calabrese & Baldwin. 1999, Hotchkiss. 1923, Miller et al. 1945, Morales-Fernandez et al. 2014, Woznica et al. 2013), plants (Barcelo & Poschenrieder. 2002, Belz & Duke. 2014, Calabrese & Howe. 1976, Jensen. 1907, Mattson. 2008, Migliore et al. 2003, Migliore et al. 2000, Migliore et al. 2010, Nickell. 1952, Velini et al. 2008, Xin et al. 2013), arthropods (Guedes et al. 2010, Guedes & Cutler. 2014, Zuo et al. 2013) and other animals, including humans (Gabliks et al. 1967, Hayes. 2007, Levy. 1998, Mattson et al. 2010). Thus, hormesis is considered as a general phenomenon independent of biological endpoint, environmental stressor and experimental model system (Calabrese & Baldwin. 2001).

This study is aimed to examine the nature of hormesis among resistant *Pythium* species. *Pythium irregulare* (Buisman, 1927) and *P. ultimum* (Trow, 1901) are oomycete plant pathogens that are presently classified under the kingdom Stramenopila, along with heterokont golden-brown algae (Baldauf et al. 2000). These fungus-like organisms are ubiquitous in nature and cause disease in wide varieties of plants including food crops, ornamental crops, and turfgrasses significantly

reducing overall agricultural productivity. Chemical control through a limited number of registered fungicides is the most common method employed to control *Pythium* diseases. However, frequent use of a single fungicide for extended period of time without active ingredient rotation has been the main cause for emergence of resistant isolates. Such isolates are not just resistant to fungicides but may be stimulated by low doses of fungicides. Fungicide hormesis leads to more aggressive growth, increased disease severity and mycotoxin production (Audenaert et al. 2010, Flores & Garzon. 2012, Garzon et al. 2011, Zhou et al. 2014). Evidences of hormetic stimulatory responses have been reported in the phytopathological literature. Previously, Garzon et al. observed up to 10% average increase in radial growth of a mefenoxam-resistant isolate of *P. aphanidermatum*, at a mefenoxam concentration of  $1 \times 10^{-14}$   $\mu\text{g/ml}$ . They also reported increased damping-off of geranium seedling severity of up to 61% at a mefenoxam concentration of  $1 \times 10^{-10}$   $\mu\text{g/ml}$  (Garzon et al. 2011). Flores et al. designed a protocol for detection and assessment of chemical hormetic effects in fungi and oomycetes using radial growth *in vitro* as the endpoint, and demonstrated significant hormetic responses in a mefenoxam and propamocarb resistant *P. aphanidermatum* isolate using subtoxic doses of propamocarb, cyazofamid and ethanol (Flores & Garzon. 2012).

Although extensively ignored by the phytopathological scientific community, the consequences of chemical hormesis in plant pathogens like *Pythium* spp. may have severe negative impacts on plant health and agricultural productivity. Thus, it is imperative to understand the effects of subtoxic doses of fungicides prior to application, create awareness among plant pathologists and growers, and incorporate the concept of hormesis as a part of integrated pest management strategies. The objective of this study was to investigate the low dose effect of mefenoxam, a systemic fungicide that inhibits protein synthesis in oomycetes, on resistant isolates of *P. irregulare* and *P. ultimum*. Hormetic effects were evaluated by measuring the growth area and dry weight of mycelia in nutrient rich and nutrient limited media.

## MATERIALS AND METHODS

***Pythium* isolates:** One isolate each of *Pythium ultimum* (P81) and *P. irregulare* (P64) with history of mefenoxam resistance were used in this study. *P. irregulare* and *P. ultimum* isolate P81 were originally isolated from geranium and viola plants respectively, exhibiting symptoms of root rot. These resistant isolates ( $EC_{50} = 100 \mu\text{g/ml}$ ) were provided by Dr. Gary Moorman (Pennsylvania State University) and stored in the dark at room temperature, in glass flasks with sterile water containing water agar (WA) plugs with oospores.

**Mycelial culture preparation:** Clean culture for each experiment were prepared by transferring stored WA plugs onto Petri dish containing the selective PARP media (Jeffers. 1986). A 5 mm diameter plug of mycelium from the emerging hyphal tip of culture was then transferred to corn meal agar (CMA) and incubated in the dark for 48 hours at 25°C. For each trial, the same method was repeated to obtain freshly prepared culture.

**Preparation of fungicide concentrations and determination of benchmark doses (BMD):** Technical-grade mefenoxam (Ridomil Gold®EC, 96.2% active ingredient, Novartis Crop Protection, Inc.) was used in the study. Evaluation of stimulatory responses to sub-lethal dose of mefenoxam was performed in two steps as described by Flores & Garzon (2012) with modifications. Solutions with different concentration of fungicides were prepared in amber bottles via serial dilutions with sterile water. The minimum application rate (MAR) of mefenoxam as recommended in the fungicide label (2.5 Kg. ai. /h) was initially used to prepare solution with different concentrations of mefenoxam. The MAR value (10.425  $\mu\text{g/ml}$ ) was estimated in  $\mu\text{g/ml}$  assuming soil depth and soil bulk density to be 2 cm and 1.2  $\text{g/cm}^3$ , respectively (Chen et al. 2001). First, six concentrations of mefenoxam ranging from  $\text{MAR} \times 10^3$  to  $\text{MAR} \times 10^{-3}$  in 10 fold dilutions and a control (distilled water) were prepared to determine the benchmark dose or BMD. BMD is a concentration close to the no observable adverse effect level or NOAEL. The BMD was calculated



by performing continuous Hill model using default parameters via Benchmark Dose Software (BMDS, V. 2.1), provided on-line by the United States Environment Protection Agency (<http://www.epa.gov/ncea/bmds/progreg.html>) as described by Flores & Garzon (2012). The BMD value for both *Pythium* species were determined to be approximately 100 µg/ml. Second, BMD value were used to assess presence of any stimulatory response in area and dry weight of mycelium. Ten different concentrations of mefenoxam (i.e. BMD x 10<sup>2</sup>, BMD x 10, BMD x 10<sup>0.5</sup>, BMD, BMD x 10<sup>-0.5</sup>, BMD x 10<sup>-1</sup>, BMD x 10<sup>-1.5</sup>, BMD x 10<sup>-2</sup>, BMD x 10<sup>-2.5</sup>, BMD x 10<sup>-3</sup>, and a water control) were prepared as a 10X stock solutions for the assays.

**Effect of sub-lethal doses of mefenoxam on mycelial growth area *in vitro*:** Corn Meal Agar (HiMedia Laboratories, LLC, NJ) was prepared as per manufacturer's instruction (17 g/l) and placed in a water bath at 60°C. The media were allowed to cool for two minute and mixed with the different 10X stock solutions for another two minutes using magnetic stirrers. The mixture were poured into 9 cm Petri dish and allowed to solidify. Each Petri dish was precisely dispensed with 20 ml media using an accu-jet® pro Pipette Controller (BrandTech® Scientific, CT). Plugs 5mm diameter of mycelium margins were transferred to the media amended with different concentration of fungicides. The plates were sealed with parafilm and placed in dark incubation chamber at 25°C. After 24 hours, Petri dish were scanned using CanoScan 8400F (Canon, Melville, NY) and the whole area of the colony was measured using KLONK Image measurement software (KLONK, Ringsted, Denmark). Each Petri dish was considered as one experimental unit and three replicates were used for each concentration. Overall, 10 concentrations (1000, 100, 31.6, 10, 3.16, 1, 0.31, 0.1, 0.03, 0.01 µg/ml) and a control (0 µg/ml) were used for each experiment, and the experiment was repeated 6-10 times for each isolate.

**Effect of sub-lethal dose of mefenoxam on dry weight of mycelium *in vitro*:** Potato Dextrose Broth (HiMedia Laboratories, LLC) was prepared according to the manufacturer's instruction (24 g/L). 20 ml of PDB media was poured in several 250 ml flask using accu-jet® pro

Pipette Controller and sterilized. The 10X stock solutions with similar concentrations as mentioned before were mixed with PDB, and one 5mm diameter of agar plug from the emerging hyphal tips of culture was transferred to each PDB flask and sealed with parafilm oil. To ensure proper mix of fungicide solution with media, each flask were placed on a rotary shaker (100 rpm) and incubated in the dark at 25°C. After 7 days, the mycelia were harvested and pressed between two filter paper to remove excess liquid. The mycelium was peeled off the filter paper, folded and placed into 2 ml Eppendorf tubes and lyophilized. The dry weight of mycelium was then measured after 48 hours. Each PDB flask was considered as one experimental unit and four replicates were done for each concentration. Overall, 10 concentrations (1,000, 100, 31.6, 10, 3.16, 1, 0.31, 0.1, 0.03, 0.01 µg/ml) and a control (0 µg/ml) were used for single experiment and the experiment was repeated two times for each isolate.

**Data analysis:** Presence of significant growth stimulation was determined using modified Brain-Cousens nonlinear model as described in equation 1 (Brain & Cousens. 1989, Flores & Garzon. 2012, Schabenberger et al. 1999). Mycelial growth area was transformed to percentages relative to control. The upper limit was set to 100% ( $\alpha$  = no inhibition, determined by average of mycelial growth area of control) and the lower limit was set to 0 ( $\delta$  = total inhibition, determined by highest dose). Initially the value for  $EC_{50}$ ,  $\beta$  (Slope at  $EC_{50}$ ) and  $\gamma$  (rate of increase at low concentration) were roughly estimated based upon experimental data. Zero or negative value of  $\gamma$  (95% CI) indicated no stimulation resulting in a monotonic curve or a decreasing sigmoidal curve. However, positive values of  $\gamma$  (95% confidence interval) represented presence of significant stimulation at low doses resulting in biphasic inverted U-shaped curve above the control. Curve modeling and estimation of the  $EC_{50}$ , NOAEL (No observed adverse effect level) and MSD (Maximum stimulation dose) was analyzed using PROC-NLIN (SAS 9.2, SAS Institute, Cary, NC) using the SAS code provided in the literature (Schabenberger et al. 1999). Finally, the average

response Y at concentration X was calculated in Excel® (Microsoft, Redmond, WA) and the results were plotted against the natural log of the corresponding doses.

$$E\left[\frac{y}{x}\right] = \delta + \frac{\alpha - \delta + \gamma x}{1 + \omega \exp\left[\beta \ln\left(\frac{x}{NOAEL}\right)\right]} \dots\dots\dots \text{Equation 1}$$

Where,

$$\omega = \frac{\gamma NOAEL}{\alpha - \delta}$$

## RESULTS

*Pythium ultimum* had significant growth stimulation in terms of both area and dry weight when treated against subinhibitory concentration of mefenoxam. The estimated value of  $\beta$ ,  $EC_{50}$  and NOAEL in terms of growth area were 1.711, 95.104  $\mu\text{g/ml}$  and 25.597  $\mu\text{g/ml}$ , respectively (Table 1). The value of  $\beta$ ,  $EC_{50}$  and NOAEL for dry weight was 1.6605, 94.299  $\mu\text{g/ml}$  and 26.061  $\mu\text{g/ml}$ , respectively (Table 1). At 95% confidence limit, P81 showed growth stimulation of 8.61% (at MSD = 10.708  $\mu\text{g/ml}$ ,  $\gamma=1.9362$ ) in growth area (Figure 1) and 12.6% (at MSD = 10.105  $\mu\text{g/ml}$ ,  $\gamma=3.1347$ ) in dry weight of mycelium (Figure 2) relative to the control. There was a slight increase in percentage stimulation at MSD with the data obtained from dry weight compared to the mycelial growth area. None of the parameters showed significant differences between two assays. A single biphasic inverted U-shaped dose-response peak modeled from data using mycelial growth area (Figure 1) and total dry weight (Figure 2) indicated a significant stimulation at the MSD.

Like *P. ultimum*, there was a substantial increase in growth area and dry weight of mycelium in response to low dose of mefenoxam for *P. irregulare*. The estimated values of  $\beta$ ,  $EC_{50}$  and NOAEL for mycelial growth area were 1.548, 103.4  $\mu\text{g/ml}$  and 23.601  $\mu\text{g/ml}$ , respectively (Table 2). The value of  $\beta$ ,  $EC_{50}$  and NOAEL for dry weight was 1.6474, 95.5  $\mu\text{g/ml}$  and 26.524  $\mu\text{g/ml}$ , respectively (Table 2). At 95% confidence limit, P64 showed growth stimulation of 12% (at

MSD = 8.65 µg/ml,  $\gamma=3.898$ ) in area (Figure 3) and 14% (at MSD = 10 µg/ml,  $\gamma=3.587$ ) in dry weight (Figure 4) relative to the control. Thus, substantial differences between dose parameters were not noticed in terms of area and dry weight data. The data fit the modified log-logistic model that defined the shape of the dose-response curve, producing a single stimulatory peak at the MSD.

Significant differences between replicates were not observed in both assay for *P. irregulare* and *P. ultimum*. The modified Brain-Cousens model detected growth stimulation in the form of a single peak at the MSD immediately below the NOAEL for curve modelling purpose. However, growth stimulation was observed in multiple concentrations tested below BMD in some repetitions for both assays (Figure 1-4).

## DISCUSSION

Sub-inhibitory doses of mefenoxam have been reported to produce hormetic effects in *Pythium aphanidermatum*, where low doses of mefenoxam increased damping off of geranium seedlings (Flores & Garzon. 2012). Flores et al. reported stimulatory effect of propamocarb and cyazofamid in mycelial growth area growth of *P. aphanidermatum* when grown in CMA (Flores & Garzon. 2012, Garzon et al. 2011). Since both studies detected stimulatory responses to low doses of fungicides in *P. aphanidermatum* there was an imminent need to determine if such stimulatory responses could be produced in other species of *Pythium*, which was confirmed for *P. ultimum* and *P. irregulare*. This study reported that low doses of mefenoxam resulted in hormetic effect in mefenoxam-resistant isolates of *P. irregulare* (P64) and *P. ultimum* (P81). Two different mycelial endpoints were measured to assess growth stimulation: area of growth using solid cornmeal agar media and total dry mass using potato dextrose broth media.

According to Calabrese, the magnitude of hormesis is quantified by maximum stimulatory response which can be up to 60% greater than existing control. Also, the width of MSD are

generally less than 100-fold of dose range immediately below NOAEL (Calabrese & Blain. 2005). For *P. ultimum*, the stimulation at MSD was 8.61% and 12.6% for mycelial growth area and dry weight respectively (Figure 1-2). Also, the estimated MSD for area (10.7 µg/ml) and dry weight (10.10 µg/ml) were less than threefold of dose range below NOAEL (area = 25.60 µg/ml, dry weight = 26.06 µg/ml) (Table 1). Similarly, for *P. irregulare*, mycelial growth stimulation was 11.95% and 14.09% at MSD for area and dry weight (Figure 3-4). The estimated MSD for growth area (8.65 µg/ml) and dry weight (=10 µg/ml) was also less than threefold of the dose range below the NOAEL (area = 23.60 µg/ml, dry weight = 26.52 µg/ml) (Table 2). The results support the quantitative features of hormetic dose-responses as described by Calabrese based on compilation of extensive toxicological literature (Calabrese & Blain. 2005).

The mechanism underlying such stimulatory response by *Pythium* species at low dose of mefenoxam remains to be answered. Stebbing studied the effect of several metal and organometallic compounds on biomass growth of hydroid colony, *Laomedea flexuosa* (Stebbing. 1982) at different intervals of time. Based upon his findings and extensive review of several toxicological studies, he proposed that hormetic responses might occur due to immediate overcorrection at lower concentration of stressor represented by strong stimulatory peaks greater than control. The initial overcorrection starts to subside with higher concentration and the recovery become overwhelmed at the highest concentration resulting in inhibition (Stebbing. 1998). Another possibility was suggested by Szabadi who was investigating several biphasic responses in past pharmacological literature. He reported one stressor, particularly toxins affected two receptor subtypes, one subtype modifying a stimulatory pathway at a particular concentration, while the other subtype modified an inhibitory pathway at a different concentration. These findings acknowledged direct stimulatory responses as alternate possible reason to hormetic mechanism (Szabadi. 1977, Calabrese. 2015). In their study of the bacterium *R. erythropolis*, Malarczyk studied the effect of highly diluted doses of formaldehyde on the activity of 4-O-demethylase (associated

with accumulation of vanillic acid). Microscopic analysis revealed the presence of many enlarged vacuoles in bacterial cells during the accumulation of a large amount of vanillic acid, and their reduction along with decrease in 4-O-demethylase activity (Malarczyk et al. 2011). Based upon these findings, Malarczyk et al. suggested that living cells might have the ability to detect the presence of submolecular concentrations of biological effectors in their environment and provide a basis for a scientific explanation of hormesis. The modified Brain-Cousens model (Schabenberger et al. 1999) used in this study detected growth stimulation at the MSD below the NOAEL and this stimulatory response was used for curve modelling represented by a peak (Figures 1-4). However, consistent growth stimulation in some repetitions was observed at a subinhibitory dose of multiple mefenoxam concentrations tested below BMD. Previous studies have reported that such effect might results due to induction of DNA repair (Kushida et al.) or due to presence of antagonistic membrane receptor (Gómez-Icazbalceta et al.). Therefore, it is possible that such biphasic response results from the activity of sublinhitory stressor affecting multiple metabolic pathways.

Detection of hormetic effects is challenging work as it demands consistent experimental conditions throughout the execution of all the repetitions in a study. Assessment of fungicide induced hormetic effects in fungal pathogens may be affected by several factors including incubation temperature, morphology of pathogen and pesticide dilution particularly when working with chemistries active at very low concentrations (Garzon et al. 2011). According to Calabrese (Calabrese & Baldwin. 1997), quantitatively evaluation of hormetic effects require determination of the NOAEL; assessment of the effects of at least five equally spaced doses below the NOAEL to provide enough data for detection; and separation between doses below one order of magnitude as hormetic dose is usually within ten-fold range. Assessment of data is crucial when demonstrating hormetic effects. Crump (2001) established four measures for evaluating such stimulatory effects that included strength of evidence, soundness of data, consistency, and biological plausibility (Crump. 2001). Based upon these information, two experimental assays were designed using two

mycelial growth as an endpoint and the results from each assay was compared. There was no significant difference among parameters defining such biphasic response (Table 1-2) indicating the consistency of both assay.

Hormetic responses are often difficult to evaluate due to background noise created by initial fluctuation in responses and experimental error issues. However, hormetic responses are reproducible and can be easily differentiated from background fluctuations by using appropriate statistical analysis tools. Several parametric (Schabenberger et al. 1999) and non-parametric (Deng et al. 2000) approaches have been proposed to accurately estimate hormetic effects. In this study, the Brain Cousens model as modified by Schabenberger (Flores & Garzon. 2012, Schabenberger et al. 1999) was used to detect and estimate hormetic effects on two mycelial growth reference endpoints, total growth area and total dry mass weight, in pathogenic *Pythium* species. These endpoints allowed more precise assessment of growth stimulation, which conferred a great advantage over radial growth on solid media in terms of reproducibility of results and detection of statistical significant stimulation.

*Pythium* are ubiquitous in nature and are causal agent of many plant diseases (Horner et al. 2012). Infection is favored by high humidity and contaminated irrigation sources which triggers dissemination of pathogen via motile zoospores. Repeated use of the same fungicide over a long period of time results in development of fungicide resistance species (Moorman et al. 2002, Sanders. 1984). Sub-inhibitory doses of fungicides can be introduced to field or greenhouses through different routes including recirculatory irrigation systems, improper dose of fungicide application by farmers or subsequent dilution of fungicide due to environmental factors like sunlight and rainfall. Impact of accidental exposure of resistant *Pythium* pathogenic species to subtoxic doses of fungicides in agricultural systems is not known, but might have detrimental effects on crop productivity (Garzon and Flores, 2013). Further studies of fungicide hormesis with field and greenhouse trials will provide insights into its potential consequences. It is recommended

that hormetic stimulatory responses, when significant, should be considered as a part of disease management strategies

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TABLES

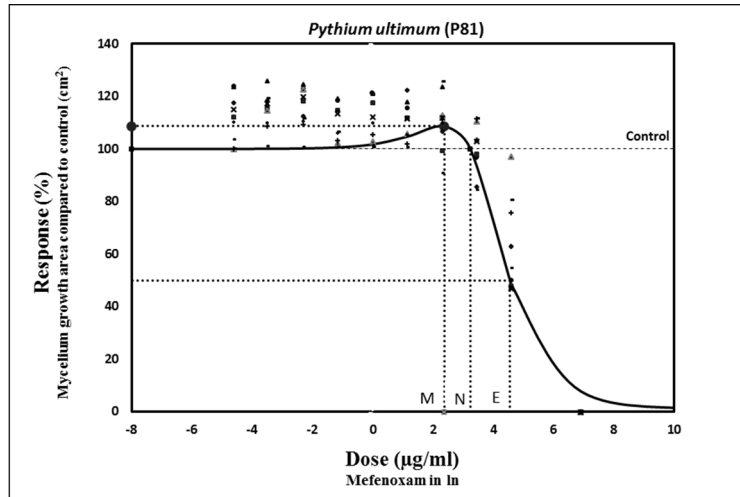
Parameter	Radial Growth (cm <sup>2</sup> )			Dry weight (mg)		
	95% Confidence limit		Estimate	95% Confidence limit		Estimate
	Upper bound	Lower bound		Upper bound	Lower bound	
<b>β</b>	1.583	1.8393	1.7111	1.3588	1.9623	1.6605
<b>γ</b>	0.9753	2.8971	1.9362	-0.3513	6.6204	3.1347
<b>EC50</b>	85.3515	104.9	95.1042	65.0018	123.6	94.2985
<b>NOAEL</b>	21.4883	29.7114	25.5974	15.9622	36.1605	26.0614
<b>MSD</b>	8.7603	12.6542	10.7075	5.2795	14.9304	10.1049

**Table 1.** Comparison of dose response parameters between area and dry weight of mycelium in response to low dose of mefenoxam in *P. ultimum*. EC<sub>50</sub>: Dose at which response is 50% of the control, NOAEL: No observed adverse effect level, MSD: Maximum stimulation dose. MSD, NOAEL and EC<sub>50</sub> are the concentration in µg/ml, β and γ represents slope at the EC<sub>50</sub> and rate of increase at low dose respectively

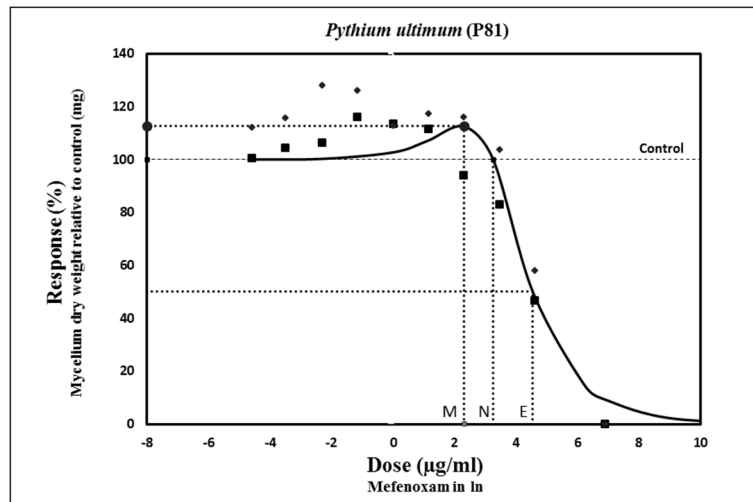
Parameter	Radial Growth (cm <sup>2</sup> )			Dry weight (mg)		
	95% Confidence limit		Estimate	95% Confidence limit		Estimate
	Upper bound	Lower bound		Upper bound	Lower bound	
<b>β</b>	1.4407	1.6563	1.5485	1.4051	1.8898	1.6474
<b>γ</b>	1.8559	5.9409	3.8984	0.4385	6.7371	3.5878
<b>EC50</b>	86.9207	119.8	103.4	69.9325	121.1	95.4121
<b>NOAEL</b>	19.069	28.1325	23.6012	18.2092	34.8387	26.524
<b>MSD</b>	6.6661	10.6445	8.6556	6.0401	13.9712	10.0056

**Table 2.** Comparison of dose response parameters between area and dry weight of mycelium in response to low dose of mefenoxam in *P. irregulare*. EC<sub>50</sub>: Dose at which response is 50% of the control, NOAEL: No observed adverse effect level, MSD: Maximum stimulation dose. MSD, NOAEL and EC<sub>50</sub> are the concentration in µg/ml, β and γ represents slope at the EC<sub>50</sub> and rate of increase at low dose respectively

**FIGURES**

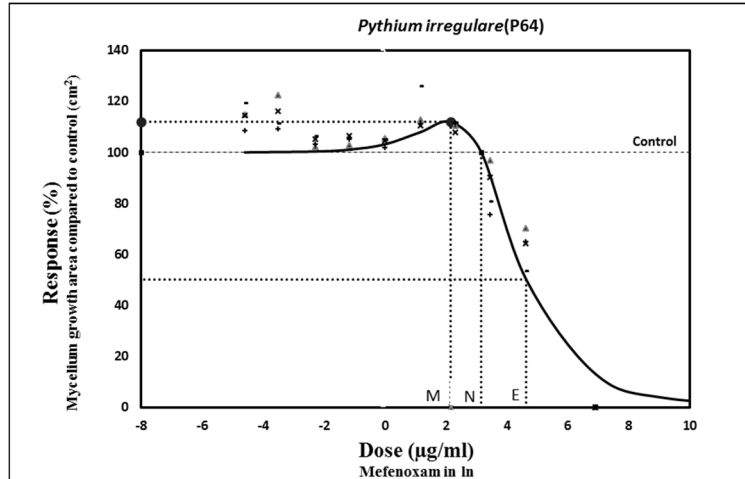


**Figure 1.** Modeled curve showing the effect of subinhibitory doses of mefenoxam on mycelial growth area in *Pythium ultimum*. Ten fungicide amended media with different concentrations of mefenoxam and a non-amended control were used for the study. The concentration of each media were converted to natural logarithm of  $\mu\text{g/ml}$ . Three replicates were used for each concentration and the experiment was repeated ten times. Growth area of each replicate was determined and expressed as percentages relative to control. Each data point in the figure represent mean value of three replicates. M, N and E represents dose value of MSD, NOAEL and  $\text{EC}_{50}$  respectively. Growth stimulation of 8.61% was observed at MSD (=10.7  $\mu\text{g/ml}$ )

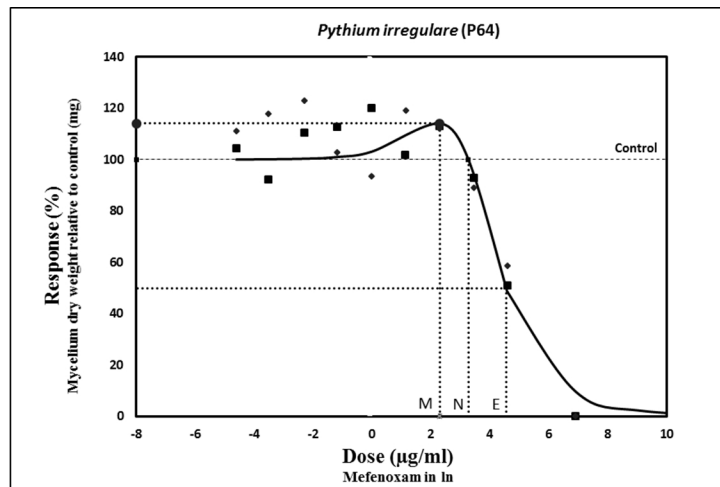


**Figure 2.** Modeled curve showing the effect of subinhibitory doses of mefenoxam on total dry weight of mycelium in *Pythium ultimum*. Ten fungicide amended media with different concentrations of mefenoxam and a non-amended control were used for the study. The concentration of each media were converted to natural logarithm of  $\mu\text{g/ml}$ . Four replicates were used for each concentration and the experiment was repeated two times. Growth area of each replicate was determined and expressed as percentages relative to control. Each data point in the figure represent mean value of four replicates. M, N and E represents dose value of MSD, NOAEL and  $\text{EC}_{50}$  respectively. Growth stimulation of 12.6% was observed at MSD (=10.1  $\mu\text{g/ml}$ )





**Figure 3.** Modeled curve showing the effect of subinhibitory doses of mfenoxam on mycelial growth area growth in *Pythium irregulare*. Ten fungicide amended media with different concentrations of mfenoxam and a non-amended control were used for the study. The concentration of each media were converted to natural logarithm of µg/ml. Three replicates were used for each concentration and the experiment was repeated six times. Growth area of each replicate was determined and expressed as percentages relative to control. Each data point in the figure represent mean value of three replicates. M, N and E represents dose value of MSD, NOAEL and EC<sub>50</sub> respectively. Growth stimulation of 11.95% was observed at MSD (=8.65 µg/ml)



**Figure 4.** Modeled curve showing the effect of subinhibitory doses of mfenoxam on total dry weight of mycelium in *Pythium irregulare*. Ten fungicide amended media with different concentrations of mfenoxam and a non-amended control were used for the study. The concentration of each media were converted to natural logarithm of µg/ml. Four replicates were used for each concentration and the experiment was repeated two times. Growth area of each replicate was determined and expressed as percentages relative to control. Each data point in the figure represent mean value of four replicates. M, N and E represents dose value of MSD, NOAEL and EC<sub>50</sub> respectively. Growth stimulation of 14.09% was observed at MSD (=10 µg/ml)

## CHAPTER IV

### THIOPHANATE-METHYL INDUCED HORMETIC EFFECT IN *SCLEROTINIA*

#### *HOMOEOCARPA*

#### ABSTRACT

The beneficial effects of subinhibitory doses of fungicides on fungal and Oomycete plant pathogens have already been reported and such non-target effect of fungicides are often termed as “fungicide hormesis” leading to triggered response in pathogens including increased growth rate, pathogenicity and mycotoxin production. Therefore, it is imperative to understand the nature of fungicide hormesis to ensure crop productivity as the consequence of overlooking such effect might be detrimental. Twenty eight isolates of *S. homoeocarpa*, causal agent of dollar spot disease in turf, were assessed for fungicide hormesis at sublethal concentration of thiophanate-methyl. Each isolate was grown in corn meal agar amended with eleven concentrations of thiophanate-methyl and the area of mycelial growth was determined relative to control. Three replicates were used per concentration and the experiment was repeated 3-5 times for each isolate. All 5 baseline isolates ( $EC_{50} > 20$  ppb), with no prior history of thiophanate-methyl exposure, were highly sensitive and did not produce any stimulation. Likewise, no stimulation was observed in two exposed-sensitive isolates ( $EC_{50} > 30$  ppb). Seventeen out of 21 resistant isolates ( $EC_{50} = 294-1550$  ppb) showed statistically significant growth stimulation in the range of 2.8-19.7% relative to control representing ubiquitous nature of growth stimulation among these group. Nonetheless, four resistant isolates lack stimulation indicating that not all resistant isolates are capable of producing fungicide hormesis. Our results suggest that hormetic effects are not common in sensitive isolates; however, 81% of resistant isolates showed significant growth stimulation indicating ubiquitous nature of hormesis among these groups.

Keywords: fungicide hormesis, Oomycete, fungal, low-dose, growth stimulation, thiophanate-methyl.

## INTRODUCTION

*Sclerotinia homoeocarpa*, F.T. Bennett (1937), is an ascomycete plant pathogenic fungus that causes dollar spot disease in more than 40 plant hosts, the majority of which belong to a grass family Poaceae (Bennett. 1937, Monteith & Dahl. 1932). Dollar spot symptoms can include hour glass shaped, chlorotic lesions on individual grass blades, and as the disease progresses, unsightly patches of the size of a silver dollar are formed in the turf surface (Smiley et al. 1992). Management with fungicides is the foremost method used to control dollar spot (Walsh et al. 1999). Although, many fungicides with different modes of action are commercially available, frequent use of fungicides on turf throughout the growing season have resulted in development of resistance to almost all groups of fungicides registered today (Bishop et al. 2008, Cole et al. 1968, Cole et al. 1974, Detweiler et al. 1983, Goldberg & Cole. 1973, Golembiewski et al. 1995, Hsiang et al. 2007, Hsiang et al. 1997, Jo et al. 2004, Massie et al. 1968, Nicholson et al. 1971, Warren et al. 1974).

Large quantity of fungicides are sprayed every year to control dollar spot in golf courses making it one of the most expensive diseases of turfgrass (Vargas. 2005). Although repeated application of fungicides over time is recommended; factors like sunlight, rainfall, decomposition of active ingredients result in exposure to lower doses of fungicides in the environment. *In vitro* studies revealed that the effect of low doses of fungicides in fungal and Oomycete pathogens trigger increased growth, longevity, mycotoxin production and tolerance (Audenaert et al. 2010, Flores & Garzon. 2012, Garzon et al. 2011, Zhou et al. 2014); such induced response is often correlated with the term hormesis. Hormesis is a toxicological concept characterized by beneficial effect in the biology of an organism resulting from exposure to sub-inhibitory doses of a stressor (chemical or radiation) which is otherwise toxic at higher doses (Calabrese & Baldwin. 2002). Hormetic effect in pathogens resulting from subinhibitory doses of fungicide is referred to as “fungicide hormesis” (Garzon et al. 2011). Direct stimulation (Calabrese. 2013, Szabadi. 1977) or initial overcorrection

due to disruption in homeostasis (Stebbing. 1982) are often considered the two possible mechanism underlying hormesis.

Few cases of fungicide hormesis have been reported for economically important fungal pathogens in recent phytopathology literature. Baraldi et al. (2003) reported improved germination for some thiabendazole (TBZ) resistant isolates of *Penicillium expansum* when exposed to subinhibitory dose of TBZ. Audenaert et al. (2010) showed that low dose exposure of prothioconazole led to a higher production of mycotoxin deoxynivalenol (DON) production *in vitro* and *in planta* by *Fusarium graminearum*. Significant radial growth stimulation was observed in *Lyophyllum palustre* and *Sclerotinia sclerotiorum* when exposed to sublethal doses of propamocarb and dimethachlon, respectively (Landry et al. 2011, Zhou et al. 2014). Zhou et al. (2014) also found increased virulence in detached oilseed rape plants after spraying with dimethachlon at a concentration of 2 µg/ml. Fungicide hormesis has also been reported in several Oomycete pathogens including *Phytophthora infestans* (Zhang et al. 1997), *P. undulate* (Kato et al. 1990) and *P. aphanidermatum* (Flores & Garzon. 2012, Garzon et al. 2011, Garzon et al. 2008). These findings highlight the importance of understanding the nature of fungicide hormesis and its consequences on plant health.

The objective of this study was to investigate the occurrence of the hormetic effect in 28 isolates of *Sclerotinia homoeocarpa* collected from various states of USA. Sensitivity of the 28 isolates to thiophanate-methyl, benzimidazole group of fungicide that inhibits DNA synthesis of the pathogen, were determined and their dose-response to a sub-inhibitory dose of thiophanate-methyl were quantified and contrasted.

## MATERIALS AND METHODS

**Isolates of *S. homoeocarpa*:** 28 isolates (Table 1) of *Sclerotinia homoeocarpa* collected from various geographical locations in the United States of America were provided by Dr. Lee Miller (University of Missouri) for the study. Long term storage of all isolates was prepared by transferring a plug of mycelium on a filter paper, dried and stored at -80°C. Five isolates (LWC27, LWC5, LWC10, PST4, and VGC5) had little to no history of exposure to chemical pesticides, so they were used as baseline isolates for comparison to other isolates (Ma & Tredway. 2013). The remaining 23 isolates were collected from golf courses with routine fungicide use, however, specific history of benzimidazole use was not known. These isolates were repeatedly grown for 10 generations on PDA containing thiophanate-methyl to acquire resistance; and will henceforth be called exposed isolates.

***S. homoeocarpa* culture:** Clean culture was prepared by transferring a piece of stored filter paper containing the isolate on a selective PDA medium containing 0.05 g/L each of chloramphenicol, streptomycin, and tetracycline (Ma & Tredway. 2013) for each experiment. A plug of mycelium was transferred to corn meal agar (CMA) amended with thiophanate-methyl and incubated in the dark for 72 hours at 25°C. This approach was repeated for 10 generations to obtain master culture. Finally, a 5mm plug of master culture was transferred to CMA for each trial.

***In vitro* fungicide sensitivity assay:** The sensitivity of each isolates to thiophanate-methyl (technical grade, 3336®DG LITE, 2.08 % active ingredient, Cleary Chemicals, LLC, NJ) was determined by measuring the mycelial growth area on corn meal agar (CMA) amended with six concentrations of thiophanate-methyl in 10 fold dilution (30,500, 3,050, 305, 30.05, 3.05 and 0.305 ppb) based on minimum application rate (MAR) as recommended on the label (73.23 kg. ai. / h). MAR value was transformed to ppb assuming soil bulk density of 1.2 g/cm<sup>3</sup> and an effective soil depth of 2.0 cm (Chen et al. 2001). All the isolates grew on CMA media amended with fungicide

at concentration of 3.05 ppb without any significant inhibition. Therefore, the benchmark dose (BMD: a concentration where there is no significant inhibition) for all isolates was roughly estimated to be 3.05 ppb, and was used to assess fungicide hormesis. Eleven different concentrations of thiophanate-methyl (i.e. BMD x 10<sup>4</sup>, BMD x 10<sup>3</sup>, BMD x 10<sup>2</sup>, BMD x 10, BMD x 10<sup>0.6</sup>, BMD x 10<sup>0.2</sup>, BMD x 10<sup>-0.2</sup>, BMD x 10<sup>-0.6</sup>, BMD x 10<sup>-1</sup>, BMD x 10<sup>-1.4</sup>, BMD x 10<sup>-1.8</sup>) and a control (without fungicide) were prepared for the assay.

**Effect of subinhibitory doses of thiophanate-methyl on fungal mycelial growth:** The experimental design for detecting fungicide hormesis was performed according to Flores & Garzon (Flores & Garzon. 2012) with modifications. 20 ml of CMA (HiMedia Laboratories, LLC, NJ) mixed with different concentrations of thiophanate-methyl were precisely dispensed in 9 cm Petri dish using accu-jet® pro Pipette Controller (BrandTech® Scientific, CT). Five millimeter plug were added to each plate from the emerging hyphal tip. The plates were sealed with Parafilm and incubated in dark at 25°C. After 48 hours, a high quality image of the plates displaying mycelial growth were captured using CanoScan 8400F (Canon, Melville, NY). KLONK Image measurement software (KLONK, Ringsted, Denmark) was used to measure the whole area of the colony. The scale for the software was calibrated with a scanned picture of a ruler. A 1-cm-line was drawn on the ruler and this scale was used throughout the experiment to ensure uniformity of calculations. Each Petri dish was considered as the experimental unit. Three replicates were done for each concentration. Overall, 11 concentrations (30,500, 3,050, 305, 30.5, 11.94, 4.75, 1.89, 0.75, 0.3, 0.119, 0.047 ppb) and a control (0 ppb) with three replicates for each concentration were used and the experiment was repeated 3-6 times for each isolate.

**Data analysis:** Statistical analyses for detection of significant stimulation and estimation of hormetic parameters were conducted using modified Brain-Cousens model as defined in equation #1 (Brain & Cousens. 1989, Flores & Garzon. 2012, Schabenberger et al. 1999). Only data showing growth stimulation was used for analysis. Model parameters were estimated using

non-linear modeling procedure PROC-NLIN (SAS 9.2, SAS Institute, Cary, NC) and the associated graphs were created with Excel®.

$$E\left[\frac{y}{x}\right] = \delta + \frac{\alpha - \delta + \gamma x}{1 + \omega \exp\left[\beta \ln\left(\frac{x}{NOAEL}\right)\right]} \dots\dots\dots\text{Equation 1}$$

Where,

$$\omega = \frac{\gamma NOAEL}{\alpha - \delta}$$

E(y/x) = expected value of the response y at concentration x:  $\alpha$  = % average mycelial growth area without concentration:  $\delta$  = % average mycelial growth area with highest concentration:  $\beta$  = slope at the EC<sub>50</sub> dose response curve:  $\gamma$  = rate of growth increase at low concentrations. NOAEL: no observed adverse effect level.

**Sensitivity of exposed isolates:** The resistance of exposed isolates was scored by dividing the EC<sub>50</sub> of each isolate by mean EC<sub>50</sub> values of all baseline isolates; these scores were referred as resistance factor (RF) for the purpose of this study. Higher RF values represent higher level of resistance. Based upon the RF value, the exposed isolates were further classified as sensitive (RF <10) and resistant (RF ≥ 10).

**RESULTS**

**Baseline Isolates:** Isolates LWC27, LWC5, LWC10, PST4, and VGC5 were not exposed to any form of chemical pesticides so they were used as baseline isolates. At 95% confidence (CI), the estimated value of  $\gamma$  for all five baseline isolates was negative indicating lack of stimulatory response to low doses of thiophanate-methyl (Table 1). Dose-response were evaluated at 30,500, 3,050, 305, 30.5, 3.05 and 0.305 ppb of thiophanate-methyl concentration. Parameters  $\beta$  and EC<sub>50</sub>

were determined (Table 1) by setting  $\gamma=0$  and used for modelling a curve (Figure 2).  $EC_{50}$  values of five baseline isolates ranged from 2.58 to 11.73 ppb with a mean of 7.71 ppb.

**Exposed sensitive isolates:** Response of exposed isolates was evaluated against 11 concentrations (30,500, 3,050, 305, 30.5, 11.94, 4.75, 1.89, 0.75, 0.3, 0.119, 0.047 ppb) and a control (0 ppb). The RF values for two exposed isolates, G5 and 451ShCT76 were 0.405 and 3.53 respectively; (RF<10) they were classified as exposed sensitive. At 95% CI, neither isolates produced a significant stimulation (their estimated values for  $\gamma$  were negative). The model of the stimulation curve was estimated in a similar manner to that of the baseline isolates, by setting  $\gamma=0$  (Figure 3).

**Resistant isolates:** The RF value for the remaining 21 isolates was significantly higher (RF>10) than sensitive isolates. Parameters  $\beta$ ,  $\gamma$  and  $EC_{50}$  were calculated for each isolates. Seventeen resistant isolates showed significant hormetic response with positive value for  $\gamma$  at 95% CI (Table 1). These isolates were further analyzed to calculate NOAEL and MSD to estimate stimulatory response and curve modelling (Table 1). D3 and CHCC10 exhibited RF values of 200.7 and 38.07 respectively and were considered the most resistant and least resistant isolate among others. At MSD (= 5.3 ppb), D3 displayed 11.7% stimulation with  $\beta$  and  $\gamma$  value of 1.17 and 15.3 respectively. Similarly, CHCC10 showed 9.2% growth stimulation with  $\beta$  (=1.30),  $\gamma$  (= 4.7658) and MSD (= 8.27 ppb). A22 with  $EC_{50}$  value of 1241.3 ppb (RF = 160.8) displayed highest degree of average stimulation of 19.7% at  $\beta$  (= 1.483),  $\gamma$  (= 1.04) and MSD (= 75.35 ppb). Wide array of stimulation was found among isolates at different concentrations showing hormesis ( $\gamma$  = positive) that ranged from 2.8-19.7% at MSD (Figure 4-5). Isolate CHCC10, PhPG4, 725Shme, RE18G16, RE18G35 and RE18G26 displayed stimulation in the range of 2.8-10% (Figure 4). Isolates 363ShCT18, RE18G38, PhPG22, S088, RE18G8, 500ShCT123, A4, A22, RCC18G15, RE18G4 and D3 exhibited growth stimulation in the range of 10.1-19.7% (Figure 5). Nonetheless, four resistant isolates, PhPG9, 55ShCT173, RE18G45 and H127 did not display significant growth



stimulation (Figure 6).  $\gamma$  value for all four isolates were negative and their RF value ranged from 55.84 to 107.36.

## DISCUSSIONS

In this study, 28 isolates of *Sclerotinia homoeocarpa* with different degree of thiophanate-methyl sensitivity were used to characterize fungicide hormesis. The low dose effect of thiophanate-methyl on mycelial growth *in vitro* on CMA was determined. All five baseline isolates, with no history of fungicide exposure, were highly sensitive and did not produce growth stimulation against any sublethal concentration of thiophanate-methyl (Figure 1). Similarly, there was no significant stimulation (Figure 2) observed in two exposed sensitive isolates (RF<10). This indicates that hormesis is not common among sensitive isolates of *Sclerotinia homoeocarpa*. In 17 out of the remaining 21 resistant isolates, there was a significant growth induction, with A22 showing the highest stimulation (19.7% at MSD), thus indicating that there is a high frequency of fungicide hormesis among resistant isolates.

Several attempts have been made to understand the fundamental mechanism of hormesis. Stebbing (1998) proposed that hormetic response might have resulted from initial overcorrection at lower concentrations represented by a strong stimulatory peak greater than controls. The initial overcorrection starts to subside with higher concentration while the recovery becomes overwhelmed at the highest concentration, resulting in inhibition (Stebbing. 1998). In this study, the growth stimulation in exposed resistant isolates might result from the initial overcorrection due to disruption in homeostasis and ability of these resistant isolate to tolerate low dose of thiophanate-methyl. In the case of sensitive isolates, the initial overcorrection might not happen due to inability to tolerate even small doses of thiophanate-methyl. Recovery might have become overwhelmed even at the lowest concentration, suggesting the ability of the pathogen to tolerate inhibitors might

trigger the repair mechanism, thus resulting in hormesis. An alternative mechanism involves direct stimulation resulting from receptor or cell signaling pathways (Calabrese. 2015, Calabrese & Baldwin. 2002). None of these examples provides a concrete explanation to the mechanism of hormesis, thus leaving the question open for further investigation.

The generality of hormesis has been the subject of controversy in the scientific community. Calabrese & Blain (2005), based on thorough review of past toxicological studies, developed a hormesis database of approximately 5600 dose-response relationships, where more than 900 stressors including chemicals and physical agents were used to assess such effect in a wide range of biological systems including fungi, bacteria, viruses, plants and humans. They concluded that hormesis is broadly generalizable and highly reproducible and is independent of a wide spectrum of chemical stressors, biological models (microorganisms, arthropods, plants and animals) and endpoints (including growth, metabolism and longevity). Although there is some disagreement (Mushak. 2007, Mushak. 2013), the consistency of hormesis on wide array of biological models and endpoints indicates its broad generalizability.

Although hormesis is known to be highly reproducible, quantification and assessment of fungicide-induced hormesis in fungal and Oomycete pathogen requires standardization of experimental factors including growth media, fungicide concentrations, incubation temperature and deliberate testing of several doses below NOAEL at intervals smaller than one order of magnitude (Garzon & Flores. 2013). Crump (2001) suggested strength of evidence, soundness of data and biological plausibility as the key factors to be considered while evaluating hormesis. Growth stimulation in fungal and Oomycete pathogens are generally measured by taking two perpendicular diameters of the colony and averaging them to get mean of the colony diameter assuming the colony to be circular (Flores & Garzon. 2012, Zhou et al. 2014). However, this technique had some limitations in measuring irregular colonies where there are significant differences in diameter across replicates (thus affecting accuracy). In this study, we used KLONK Image measurement

software to measure the whole area of the colony; this method was more reliable while measuring the irregular colonies as in case of *S. homoeocarpa*. Our results reported that 81% of the resistant isolates produced significant stimulation showing high frequency of fungicide hormesis among these groups. However, four exposed resistant isolates did not displayed significant stimulation suggesting that hormesis might not be present in all resistant isolates. Isolate D3 with highest resistant factor displayed growth stimulation less than other isolates (Figure 8) indicating that higher resistant might not result in higher stimulation. Overall, pooled modeled curve of 28 isolates displayed wide range of response for all thiophanate-methyl concentrations indicating variation in response at intra-species level of *S. homoeocarpa* (Figure 7).

Although the study of hormetic effect in the field environment is limited, evidence of fungicide hormesis in a wide range of pathogens in laboratory experiments highlight the importance of fungicide hormesis and its effect in plant pathogens. Hormetic effect in the form of growth stimulation, increased virulence and higher level of mycotoxin production at low dose of fungicides has already been reported in fungal (Audenaert et al. 2010, Baraldi et al. 2003, Landry et al. 2011, Zhou et al. 2014) and Oomycete (Flores & Garzon. 2012, Garzon et al. 2011, Zhang et al. 1997) pathogens. Extended research in field environments is required to understand the occurrence and the quantitative effect of hormesis in plant pathogens, especially in the case of fields with a history of pathogen resistance. Presence of such effects may increase plant damage, resulting in higher disease incidence and severity. It is therefore recommended to consider the concept of “fungicide hormesis” as part of fungal and Oomycete disease management strategies for effective management of crops.

## **ACKNOWLEDGEMENTS**

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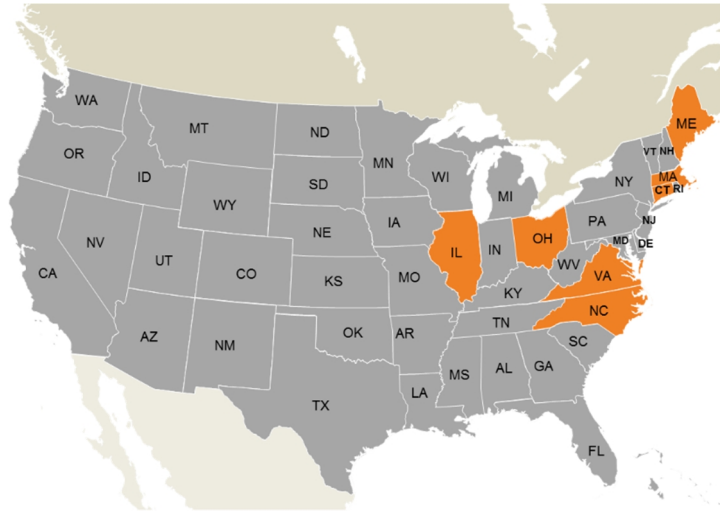
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## TABLES

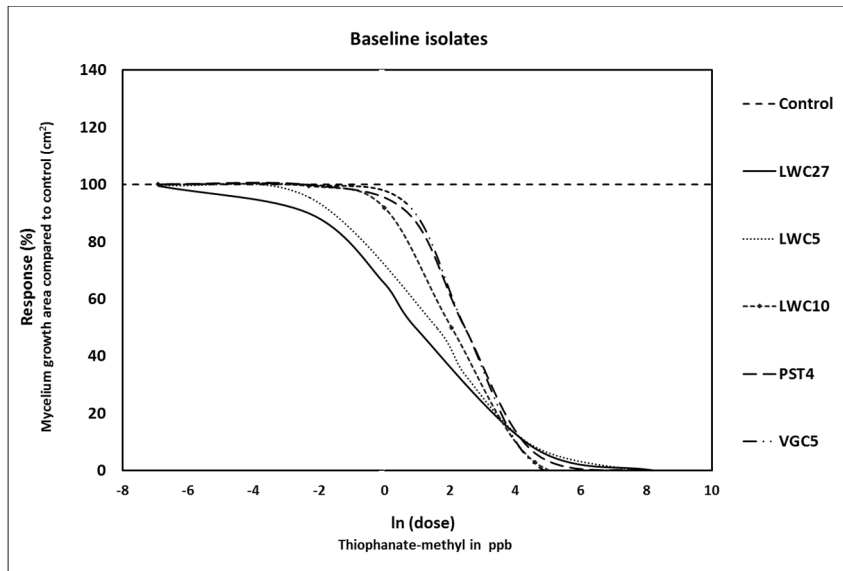
Isolate	Id	Origin	Year	Mgmt	T-methyl exposure	Beta	Gamma	EC <sub>50</sub>	RF	NOAEL	MSD
1	LWC27	Raleigh, NC	2003	Green	Baseline	0.6729	-0.0186	2.5824	-	-	-
2	LWC5	Raleigh, NC	2003	Green	Baseline	0.7852	-0.0502	4.9058	-	-	-
3	LWC10	Raleigh, NC	2003	Green	Baseline	1.173	-0.4481	7.757	-	-	-
4	PST4	Rolesville, NC	2003	Green	Baseline	1.2647	-0.02814	11.6169	-	-	-
5	VGC5	Edgartown, MA	2007	Green	Baseline	1.4961	-0.5	11.7372	-	-	-
6	G5	Canal Winchester, OH	2003	Green	Exposed	4.5591	-2.04	3.1273	0.405098	-	-
7	451ShCT76	Madison, CT	2007	Tee	Exposed	21.987	-0.6956	27.2791	3.533626	-	-
8	CHCC10	North Attleboro, MA	2007	Fairway	Exposed	1.3025	4.7658	293.9	38.07064	26.47	8.27
9	363ShCT18	East Falmouth, CT	2006	Green	Exposed	1.1967	46.0157	365.8	47.38428	10.62	2.0502
10	PhPG9	Pinehurst, NC	2003	Green	Exposed	1.6017	-0.0323	431.1	55.84298	-	-
11	557ShCT173	Woodbridge, CT	2007	Green	Exposed	1.4798	-0.0304	482.3	62.47523	-	-
12	PhPG4	Pinehurst, NC	2003	Green	Exposed	1.4829	0.4113	606.6	78.57655	98.9254	39.3375
13	RE18G38	Shallotte, NC	2003	Green	Exposed	1.715	0.3958	641	83.03259	189.1	75.4612
14	PhPG22	Pinehurst, NC	2003	Green	Exposed	1.2318	10.7806	701.1	90.81771	34.247	7.5397
15	SO88	Chicago, IL	1993	Fairway	Exposed	1.1483	97.2556	747.2	96.78932	6.9373	1.1222
16	725ShME	South Portland, ME	2007	Fairway	Exposed	1.3532	0.7022	796.5	103.1754	87.8303	31.7776
17	RE18G8	Shallotte, NC	2003	Green	Exposed	1.6549	0.9067	796.8	103.2143	249.6	79.0243
18	RE18G16	Shallotte, NC	2003	Green	Exposed	1.4558	0.2141	798.3	103.4086	99.8091	41.4525
19	500ShCT123	Coventry, CT	2007	Tee	Exposed	1.637	0.3668	815.1	105.5848	180	71.3073
20	RE18G45	Shallotte, NC	2003	Green	Exposed	0.7881	-0.00367	822.6	106.5563	-	-
21	RE18G35	Shallotte, NC	2003	Green	Exposed	1.5843	0.2602	824	106.7377	175.8	71.4024
22	H127	Springfield, OH	2005	Green	Exposed	0.8075	-0.00385	828.8	107.3595	-	-
23	A4	Gainesville, VA	2001	Green	Exposed	1.2123	8.9181	964.9	124.9893	35.8796	8.2087
24	A22	Powell, OH	2001	Fairway	Exposed	1.4828	1.0423	1241.3	160.7931	273.1	75.3535
25	RE18G26	Shallotte, NC	2003	Green	Exposed	1.3415	0.4652	1340.7	173.669	140.5	50.2032
26	RCC18G15	Raleigh, NC	2003	Green	Exposed	1.4284	1.1394	1442.7	186.8816	266.7	70.2299
27	RE18G4	Shallotte, NC	2003	Green	Exposed	1.417	0.7101	1542.2	199.7705	262.8	78.9857
28	D3	Grove City, OH	2002	Green	Exposed	1.1695	15.2964	1549.4	200.7031	25.6456	5.2919

**Table 1.** Isolates 1-5 were baseline isolates (with little to no history of previous history of exposure to thiophanate-methyl) and remaining 6-28 were exposed isolates (resistance gained by growing on media amended with 305 ppb thiophanate-methyl for 10 generations). EC<sub>50</sub> of baseline isolates were calculated using six 10 fold dilution of thiophanate-methyl from 30500-0.305 ppb. EC<sub>50</sub> of exposed isolates were calculated using eleven concentrations: 30500, 3050, 305, 30.5, 11.94, 4.75, 1.89, 0.75, 0.3, 0.119 ppb. Modified Brain-Cousens model as described by Schabenberger et al. was used to determine dose parameters: beta, gamma, EC<sub>50</sub>, NOAEL and MSD. The values for EC<sub>50</sub>, NOAEL and MSD are in ppb. Resistant factor was calculated by dividing EC<sub>50</sub> value of each isolate by mean EC<sub>50</sub> value of baseline isolates

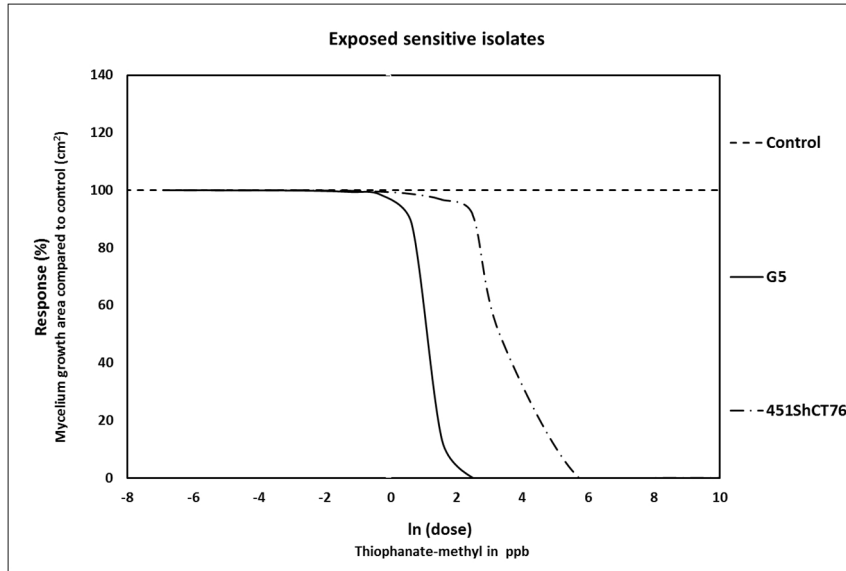
## FIGURES



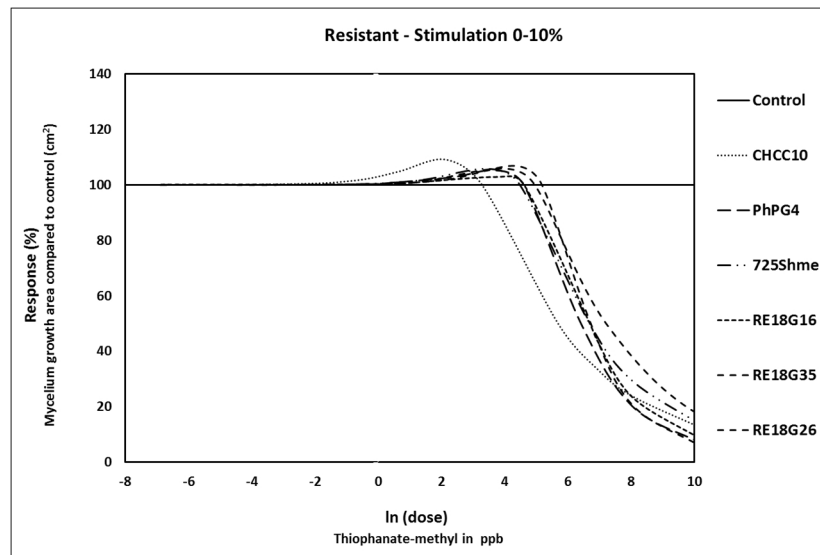
**Figure 1.** Origin of 28 isolates of *S. homoeocarpa* studied (States in orange)



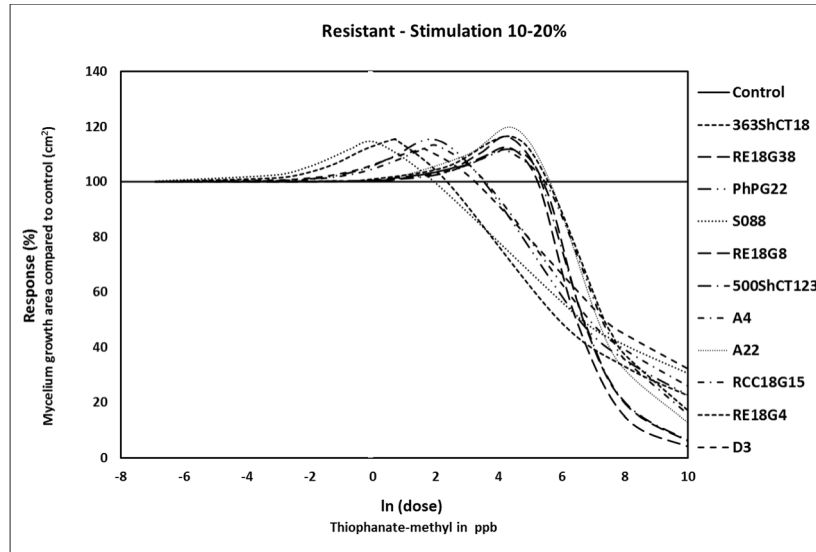
**Figure 2.** Pooled modeled curve of five baseline isolates of *Sclerotinia homoeocarpa* in response to thiophanate-methyl. Three repetitions were used per concentration and the experiment was repeated three to five times for each isolate



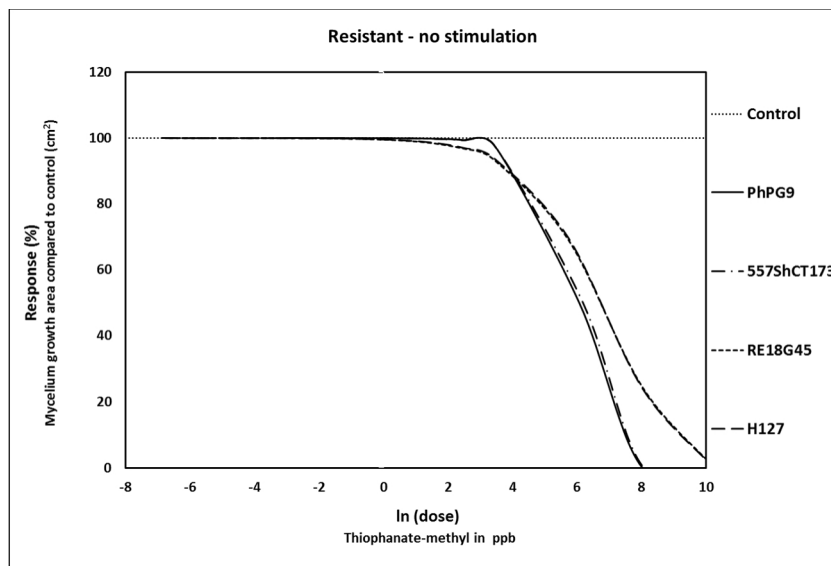
**FIGURE 3.** Pooled modeled curve of two exposed sensitive isolates of *Sclerotinia homoeocarpa* in response to thiophanate-methyl. Three repetitions were used per concentration and the experiment was repeated three to five times for each isolate



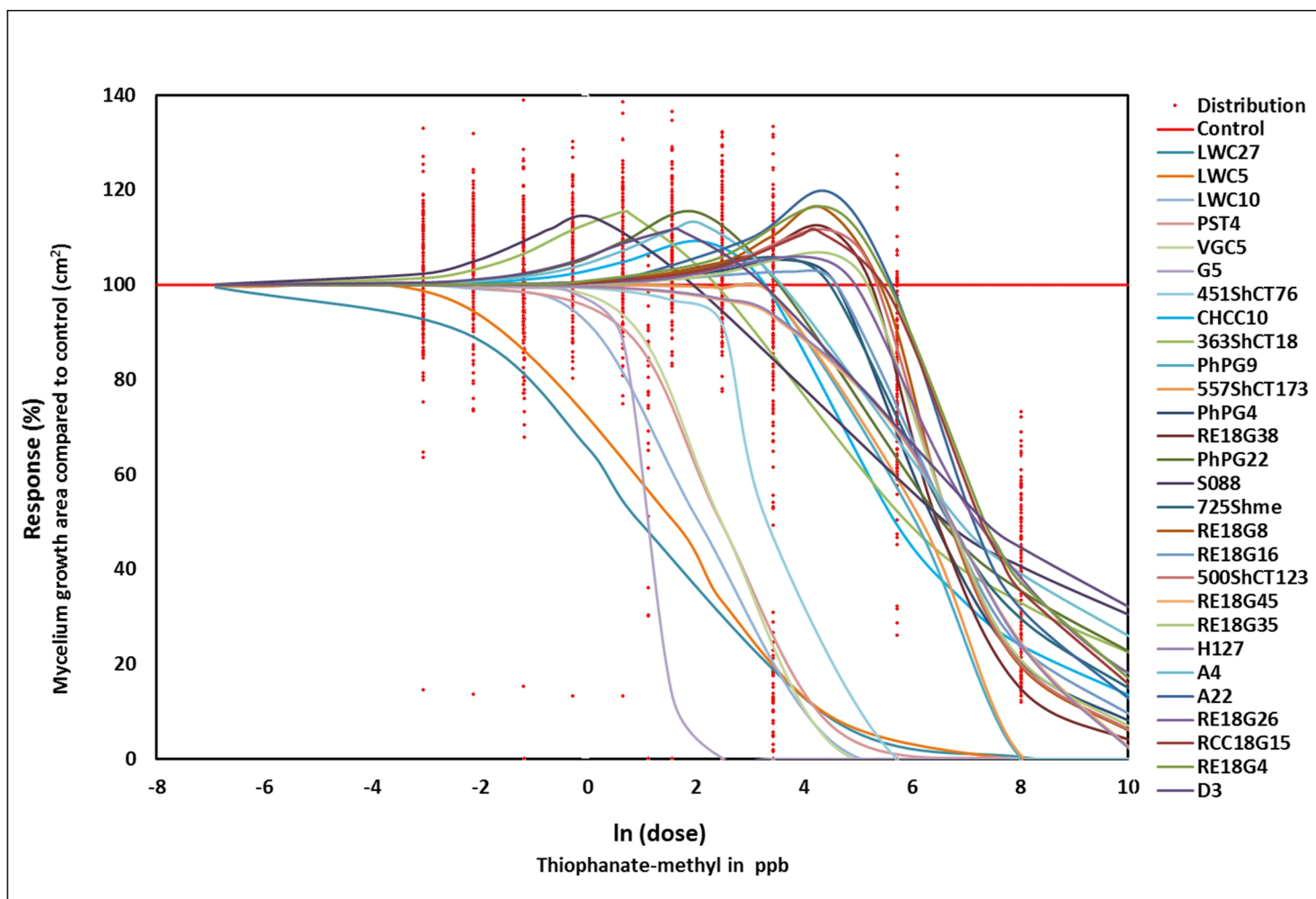
**FIGURE 4.** Pooled modeled curve of four exposed resistant isolates of *Sclerotinia homoeocarpa* in response to thiophanate-methyl showing no stimulation. Three repetitions were used per concentration and the experiment was repeated three to five times for each isolate



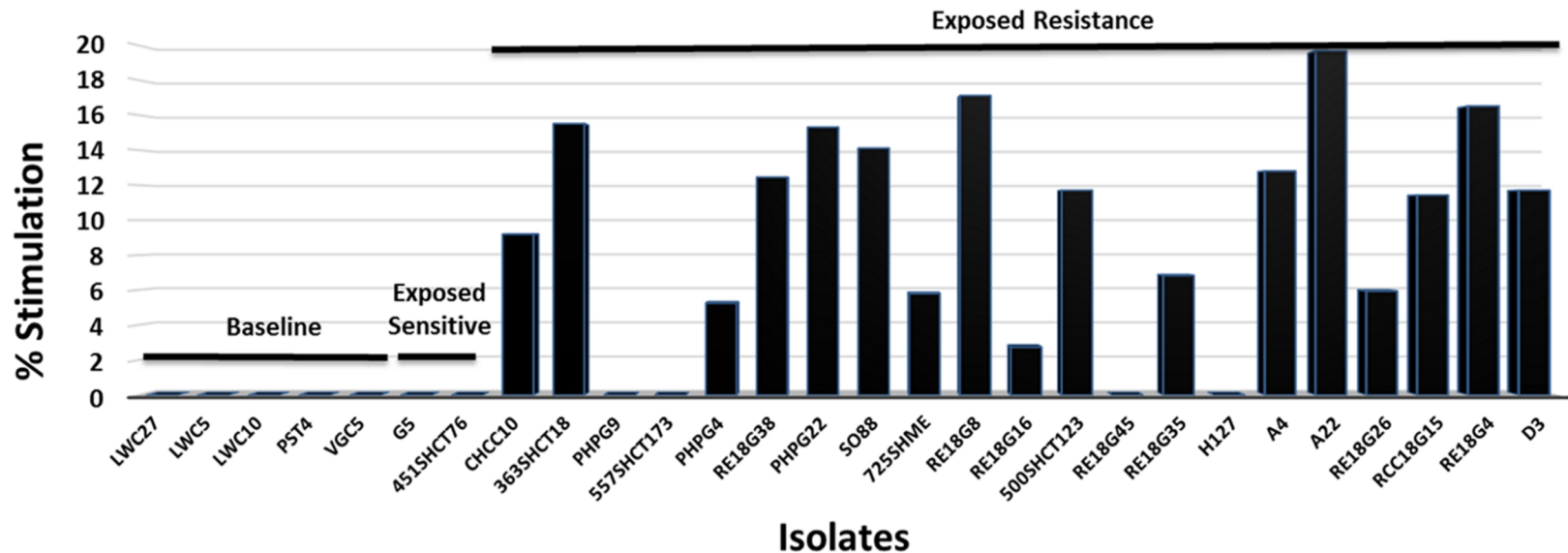
**FIGURE 5** Pooled modeled curve of six exposed resistant isolates of *Sclerotinia homoeocarpa* in response to thiophanate-methyl showing stimulation ranging from 1-10% at MSD. Three repetitions were used per concentration and the experiment was repeated three to five times for each isolate



**FIGURE 6.** Pooled modeled curve of eleven exposed resistant isolates of *Sclerotinia homoeocarpa* in response to thiophanate-methyl showing stimulation ranging from 10-20% at MSD. Three repetitions were used per concentration and the experiment was repeated three to five times for each isolate



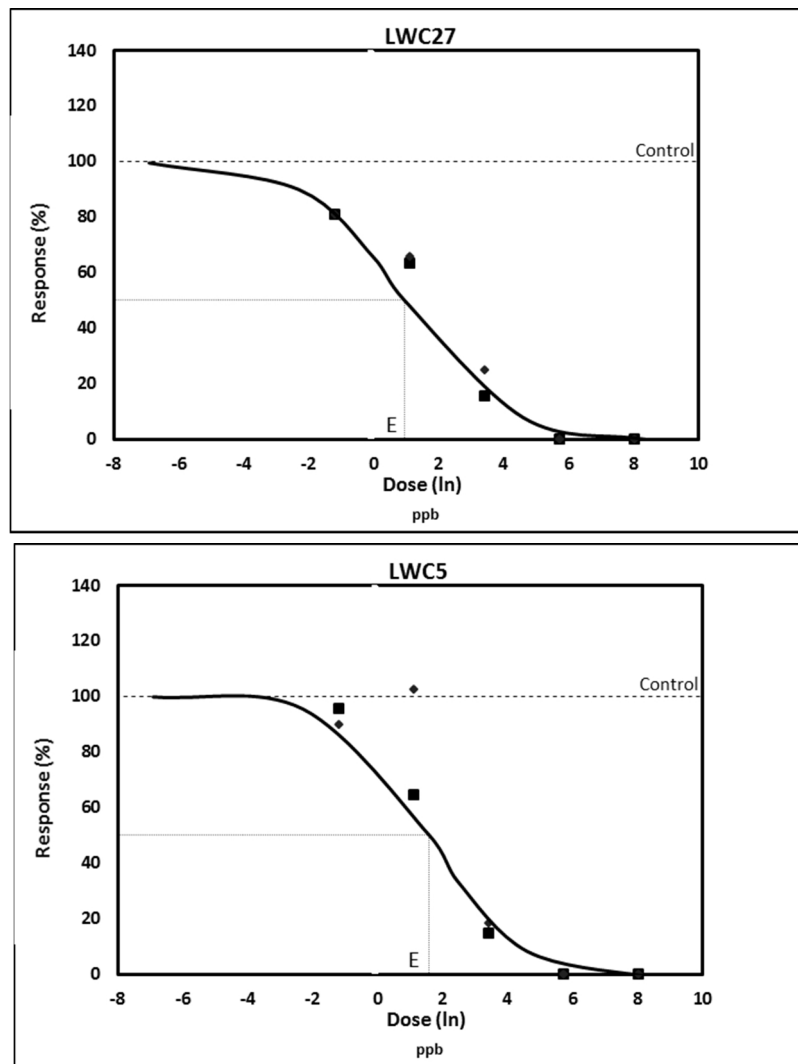
**FIGURE7.** Pooled modeled curve of 28 isolates of *Sclerotinia homoeocarpa* in response to thiophanate-methyl displaying wide range of stimulatory response at particular concentration. Three repetitions were used per concentration and the experiment was repeated three to five times for each isolate. Isolates showing stimulation at maximum stimulation dose are characterized by inverted U shaped curve above control line. Each dots represent mean response of three replicates for a single isolate at particular concentration



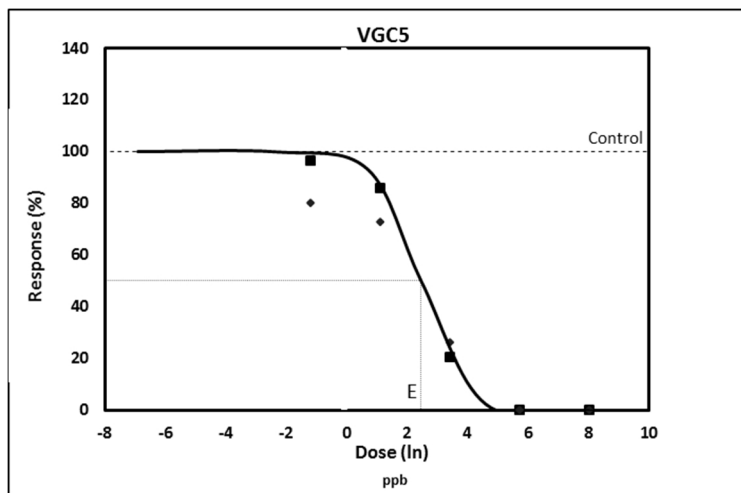
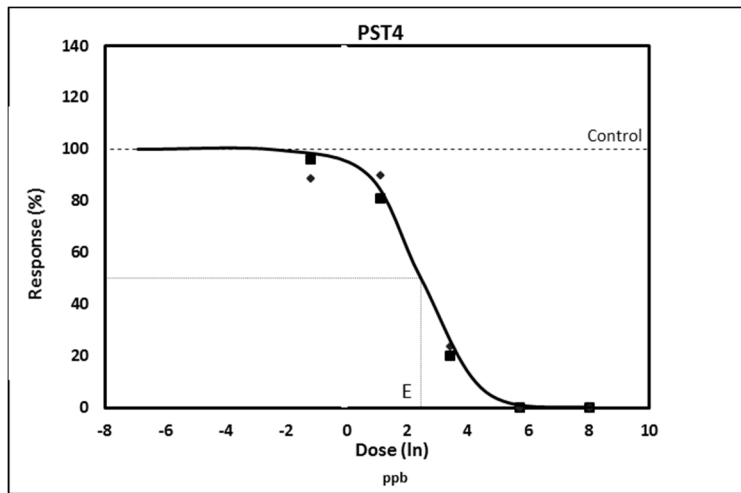
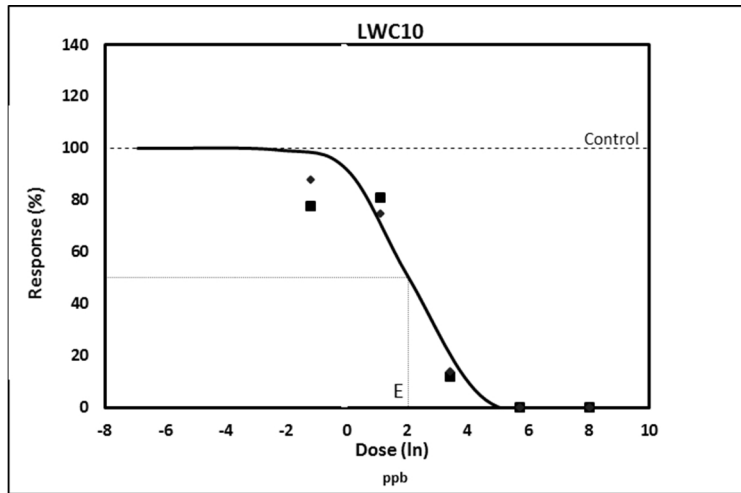
**FIGURE 8.** 28 isolates were analyzed for growth stimulation at sublethal concentration of thiophanate-methyl. Isolates were grown on solid media amended with varying fungicide concentrations ranging from 30500 – 0.047 ppb and compared with fungicide-free control. The isolates are arranged according to increasing resistant factor starting from left to right. Percentage stimulation was calculated by comparing the mean value of response at MSD with mean value of non-amended control. Overall, seventeen isolates showed hormesis with stimulation ranging from 2-20% at maximum stimulation dose. However, four resistant, two exposed sensitive and five baseline isolates did not displayed significant growth stimulation

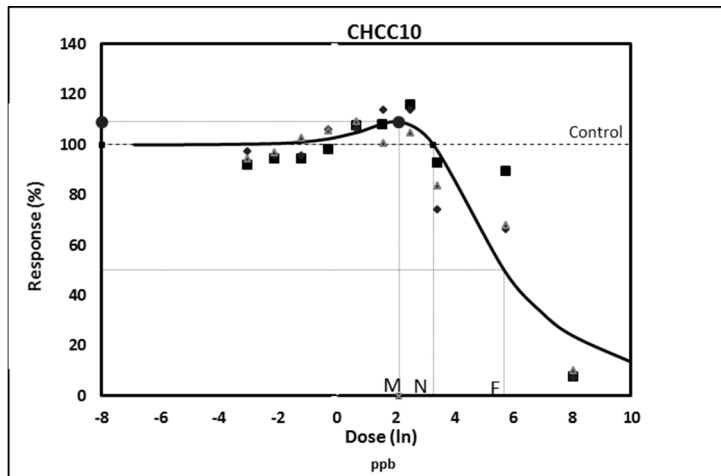
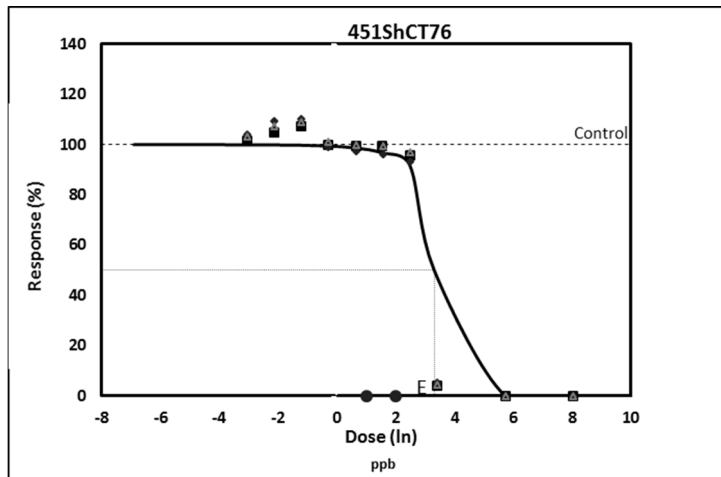
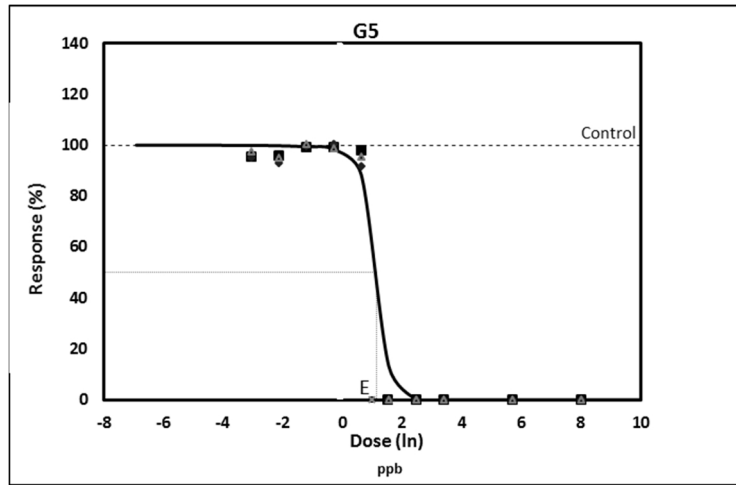
## APPENDIX

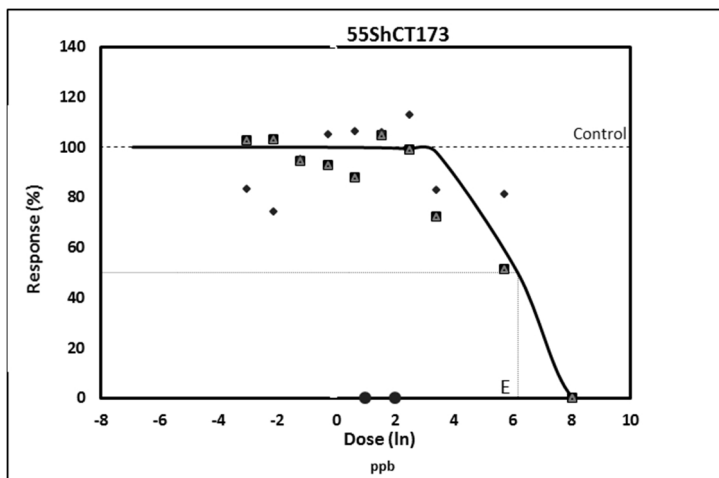
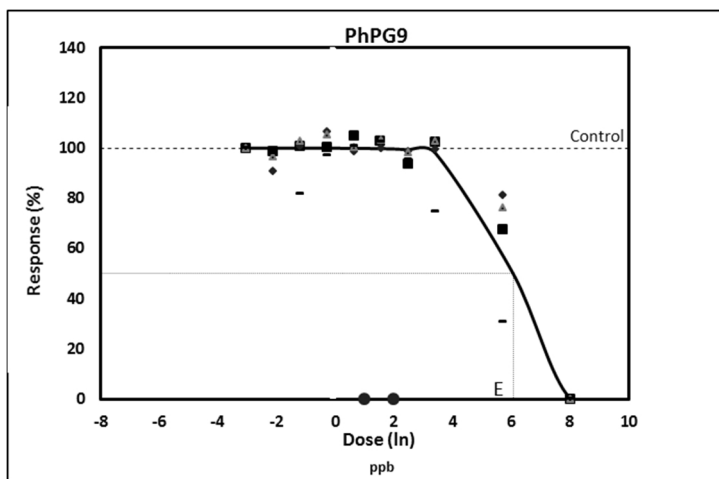
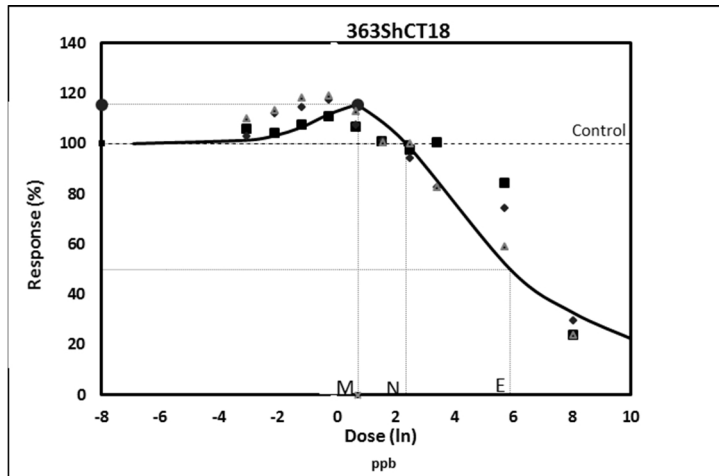
Modeled curve of each isolates of *Sclerotinia homoeocarpa* in response to eleven concentrations of thiophanate-methyl. Three repetitions were done per concentration and the experiment was repeated three to five times for each isolate. Each dots represent mean response of three replicates for an isolate at particular concentration. The concentration of each concentrations were converted to natural logarithm of ppb. M, N and E represents dose value of MSD, NOAEL and EC<sub>50</sub> respectively

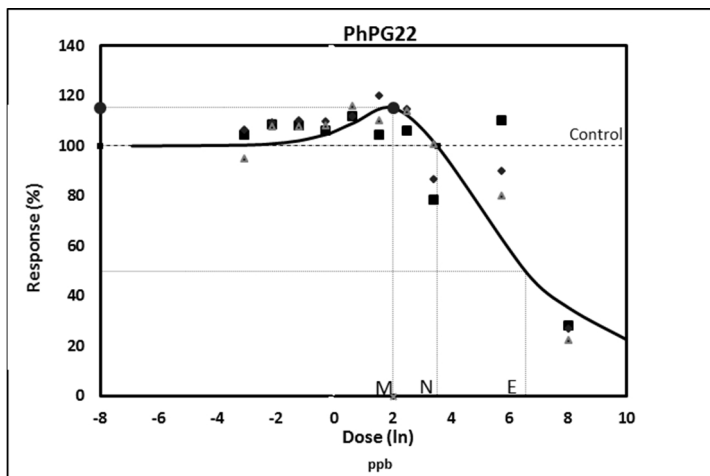
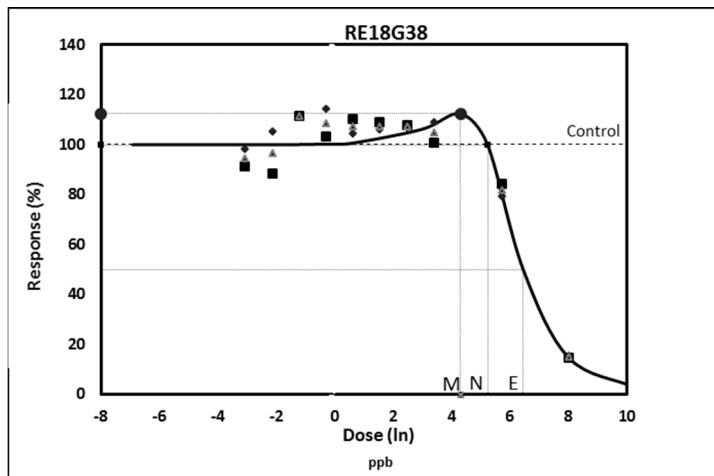
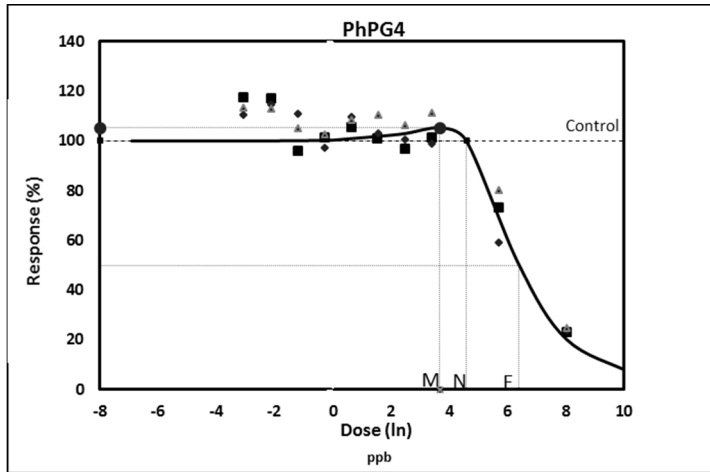


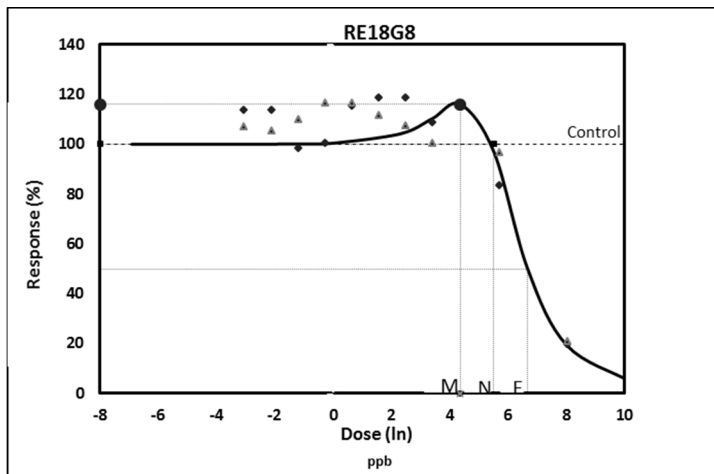
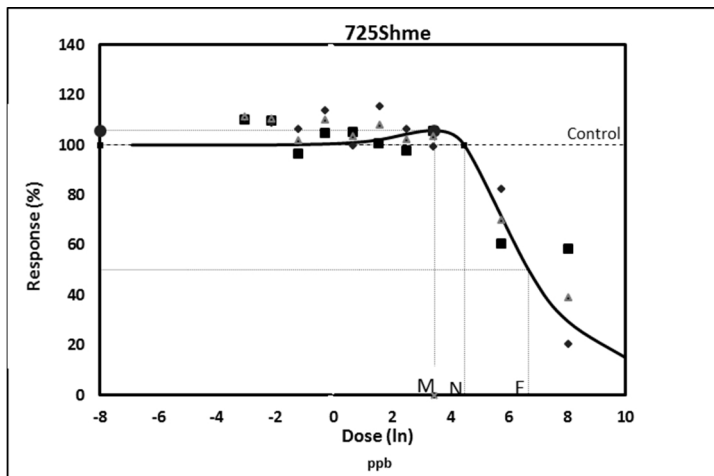
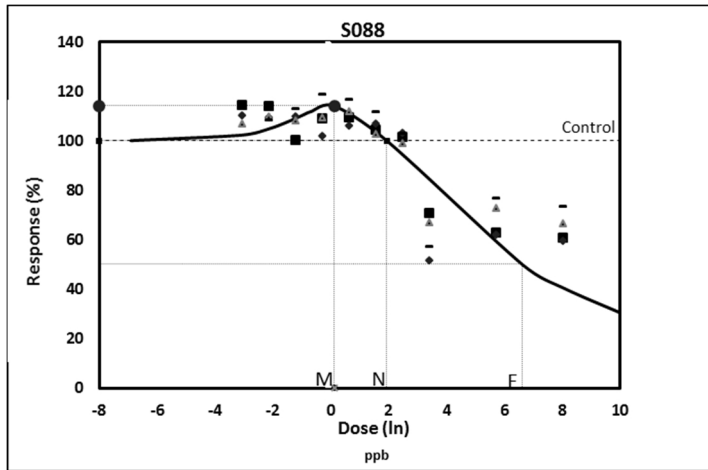


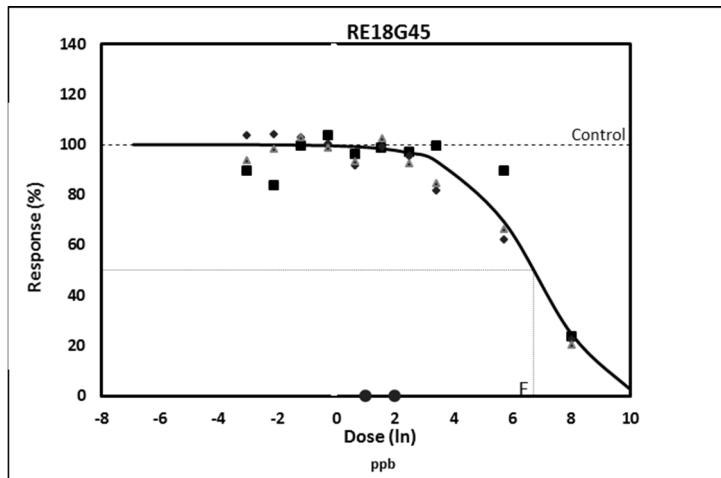
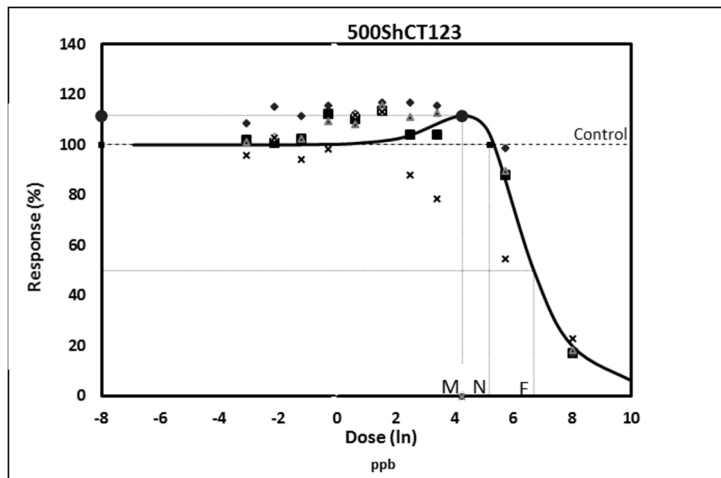
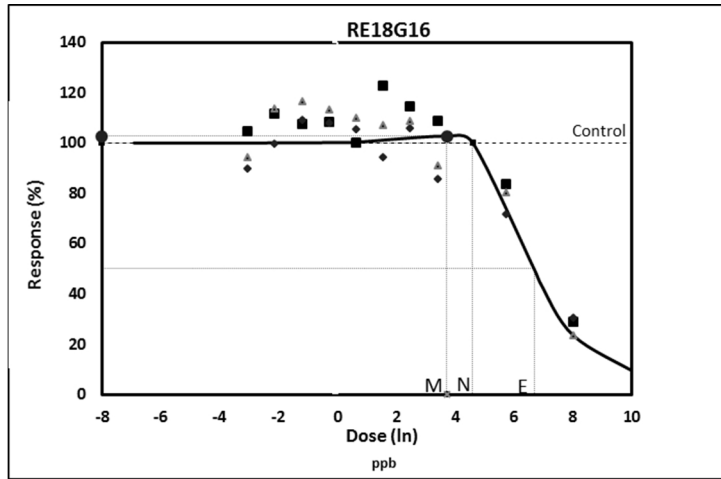


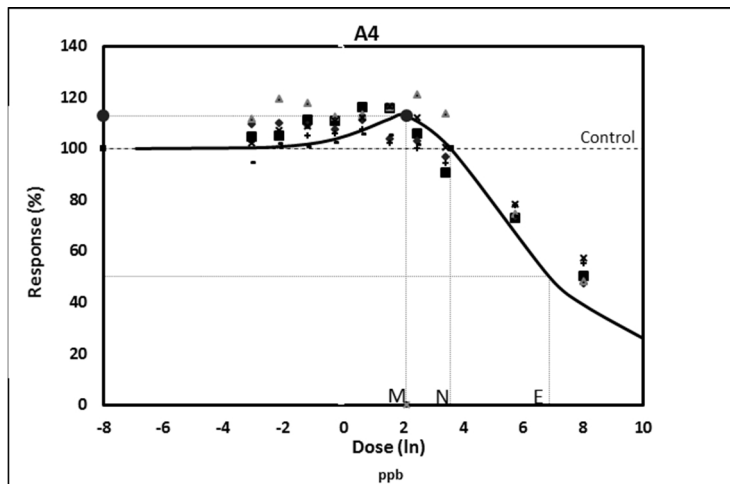
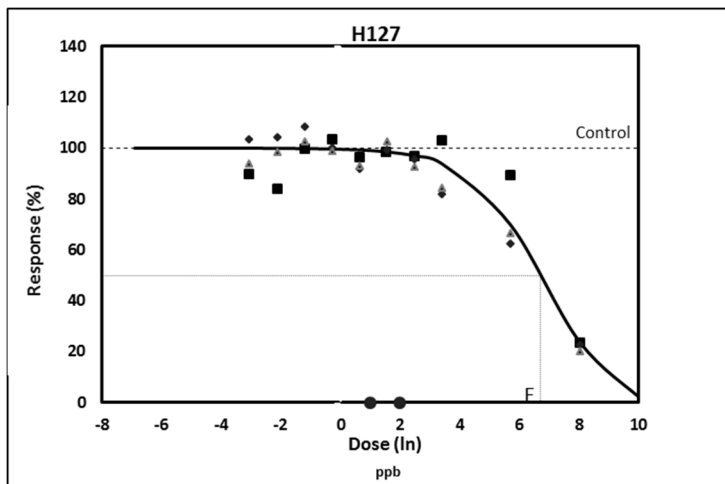
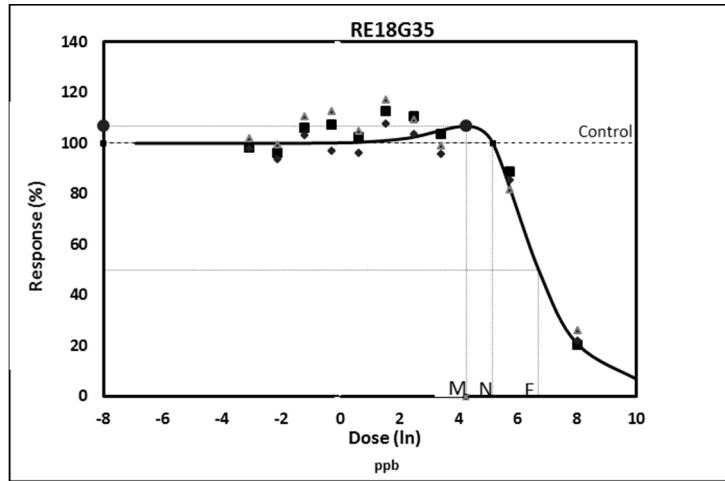


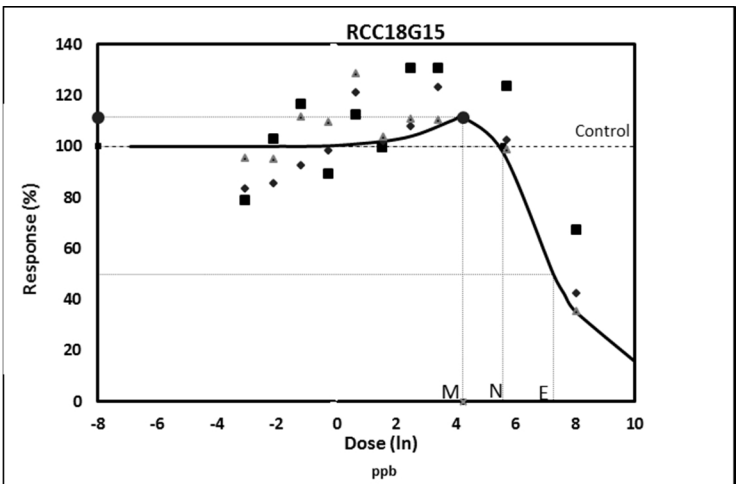
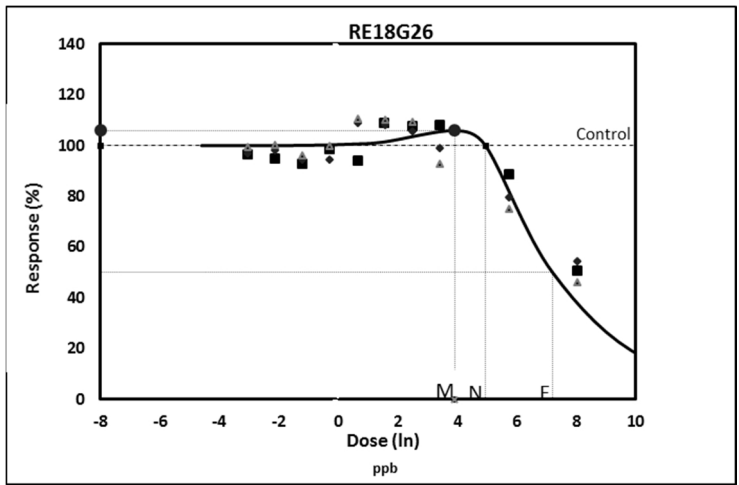
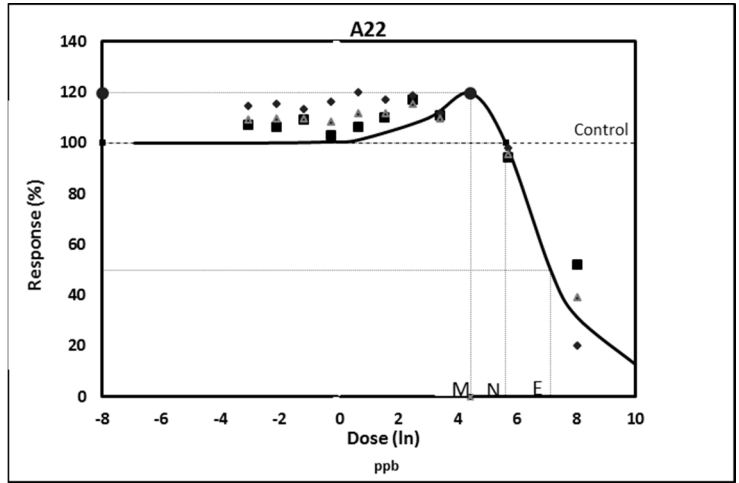




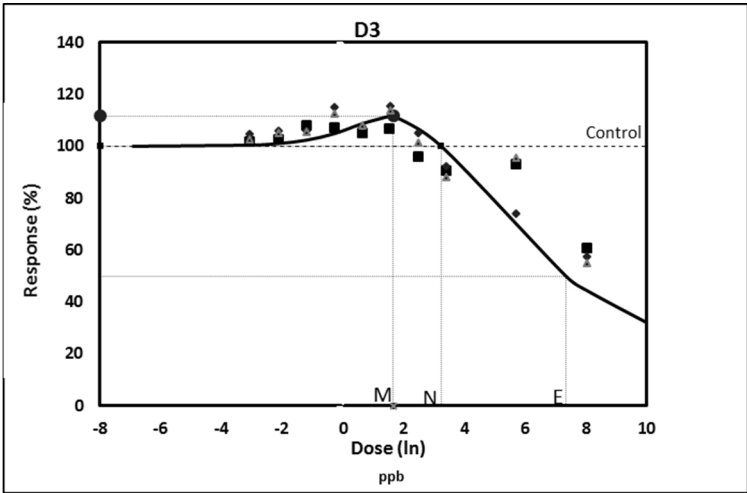
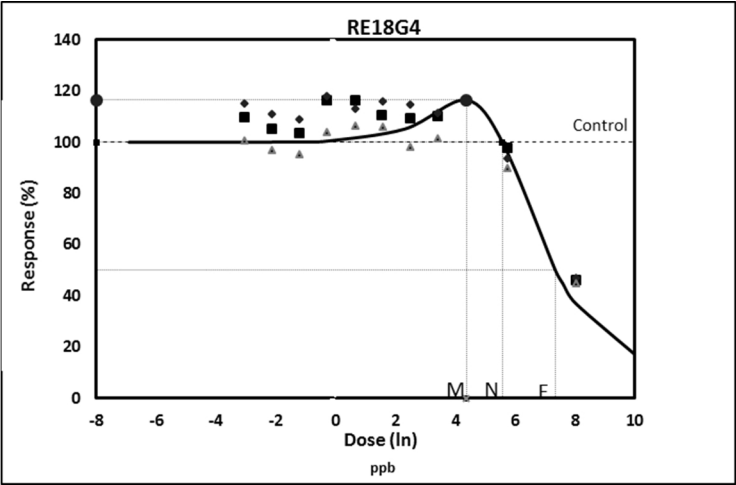












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