EVALUATING THE EFFECT OF INCREASING SALINITY ON THE GROWTH OF ROOT ROT PATHOGENS OF COMMON BEAN (*PHASEOLUS VULGARIS* L.)

By

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DEDICATION

This thesis is dedicated to my family, especially my parents who always encouraged me to continue with my college career in order to have a successful future. They have provided me with the majority of everything that has helped me end up where I am today. I also would like to thank my girlfriend Morgan, who has been supportive of me from day one in continuing school for a higher degree. This has been a long journey for my college career but I have finally made it to where I want to be. Without their persistent guidance, support, and encouraging thoughts, I would have never pushed myself this far.

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Evaluating the Effect of Increasing Salinity on the Growth of Root Rot Pathogens of Common Bean (*Phaseolus vulgaris* L.)

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is the most important food legume valued for human consumption worldwide. It provides an important supply of dietary calories, vitamins, minerals, and contains as much as 30% protein for its seed size. It is also favored as a sustainable crop due to its ability to fix atmospheric nitrogen. Abiotic and biotic stress factors are major constraints to common bean production. Among the abiotic stress factors, soil salinity is the most devastating in terms of yield loss. Soil salinity is on the rise and it affects nodulation and crop growth, which result in yield losses. There are many contributors to soil salinity including natural weathering and human interventions. Biotic stress factors include a variety of pathogens causing diseases including root rots. The most prevalent root rot pathogens of common beans are *Fusarium solani* and *Rhizoctonia solani* where they can reduce yield as much as 100%. In the field, plants are confronted with a combination of biotic and abiotic factors that can affect their growth and productivity. However, studies on the combined effects of these factors on common bean are very limited and interactions between salinity and root rot pathogens and its overall effect on common bean are unavailable. Therefore, this study was set out to understand this gap using methods to evaluate the potential effect of increased salinity on fungal development including an assessment of radial mycelial growth, fungal biomass, and spore germination *in-vitro* using solid and liquid culture media amended with sodium chloride (NaCl). Severity of rots on common bean roots were also evaluated under increasing salt stress. Significant differences in mycelial growth for both pathogens were recorded. Dry fungal biomass production in liquid media was found to be

significantly different in the NaCl-amended media for both pathogens compared to the non-amended control. Spore germination of *F. solani* was also negatively affected by the presence of NaCl in culture media. In the growth chamber experiments, the disease severity of both root rots was found to be significantly higher in the presence of the NaCl.

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CHAPTER I: INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the most important food legumes for human consumption providing adequate protein, vitamins, minerals, and fiber especially in poorer regions of Africa and Latin America (Broughton *et al*., 2003). Globally, the common bean is the most important legume species for human consumption (Schwartz & Pastor Corrales, 1989) with worldwide production over 26 million metric tons annually (FAOSTAT, 2018). Common bean is a beneficial food source, complementary to other dietary grains that contain starch and carbohydrates while providing 20-25% of protein by seed weight (Broughton *et al*., 2003). This crop is a dietary staple to over 500 million people in developing countries and complements carbohydrate rich crops such as maize, rice and cassava (Graham & Vance, 2003). It is often favored to use as a sustainable crop due to its ability to fix atmospheric nitrogen through a symbiotic relationship with rhizobia (Schmutz *et al*., 2014). In the US alone, over 2 million acres were planted with common bean in 2017 (NASS, 2017). However, common bean production is challenged by abiotic and biotic stress factors.

Common bean is sensitive to abiotic stress factors or environmental stresses including soil salinity, which is one of the most devastating to production (Shafique *et al*., 2014; Ghoulam *et al*., 2002) especially in arid and semi-arid regions (Lluch *et al*., 2007). The beginning of the 21st century experienced a global water scarcity, increased soil and water salinization (Shrivastava & Kumar, 2014) and it has been increasing at a rate of 10% annually from natural and human causes (Jamil *et al*., 2011). Soil salinity is a major threat to crop production worldwide, affecting more than one third of the irrigated land (Singh, 2015). Salt-affected soils account for as much as 20% of the total cultivated land and 30% of irrigated land area (Jamil *et al.,* 2011). Salinity reduces the ability of plants to utilize water, which induces drought that is responsible for reduced growth, metabolic processes, and yield loss. In addition to the increasing human population and decreasing

available land for farming that pose threats for our future food security and sustainability (Shabaz & Ashraf, 2013), it has been estimated that with increasing salinity, over 50% of the arable land will be affected by salt by the year 2050 (Jamil *et al*., 2011).

Biotic stress factors that affect common bean production are fungal pathogens. One such disease caused by fungal pathogens are root rots. Root rot is an expansive term that represents a variety of causative agents that cause the disease. Few common and economically important soil borne pathogens associated with root rots include *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., and *Rhizoctonia solani* (Otten & Gilligan, 2006). However, *Fusarium solani* and *R. solani* are two of the most predominant and destructive pathogens to common bean causing yield losses of 42-88% (Beebe *et al*., 1981; Tan & Tu, 1995) and as high as 86% (Abawi & Corrales, 1990). These pathogens reduce root quality, plant stand, and thereby affect yield (Henriquez *et al*., 2014). The severity of root rot and yield losses vary depending on the host response, pathogen activity, and the environmental and soil parameters (Abawi & Pastor Corrales, 1990; Naseri & Marefat, 2011; Naseri, 2013). Previous studies focused on pathogen effect alone but are lacking the combined effects of abiotic and biotic stress factors.

For this research, we aimed to evaluate how the salinity would affect these two pathogens at increasing rates *in-vitro* using solid and liquid media in laboratory experiments. Asexual reproduction is an important cycle of *F. solani* therefore we aimed to see how spores would germinate under salt stress. There is an abundant amount of literature on the effects of salinity on common bean alone but lacks the combination of stresses. Overall, we wanted to see how the disease severity would be affected on the plant by the introduction of salinity for a combination of abiotic and biotic factors.

1.1 Objectives

In order to understand the combined effects of salinity and root rot pathogens, this research was separated into four objectives.

1. To assess whether increasing salinity had a positive or negative effect on mycelial growth of *F. solani* and *R.solani* isolates
2. To evaluate the effect of increasing salinity on fungal biomass of these two fungal, root pathogens
3. To determine the effect of increased salinity on *F. solani* spore germination
4. To assess the disease development under increasing salinity

1.2 Hypotheses

The hypothesis for radial mycelial growth was that mycelial growth of these pathogens will decrease when sodium chloride (NaCl) concentrations increase compared to the un-treated control. The hypothesis for dry fungal biomass is that when the NaCl concentrations increase from the control, biomass production will decrease. The hypothesis for germination of spores under NaCl salt treatments is that the number of germinating spores, and the germination percentage will be reduced. Through the combination of abiotic and biotic factors applied to common bean plants, the hypothesis is that the disease severity will increase with the increasing NaCl content due to the weakening of the plant system under a combination stress and a higher salt tolerance of the fungal pathogens.

CHAPTER II: LITERATURE REVIEW

2.1 Common Bean

Common bean is one of the most important protein sources to humans and livestock around the world (Castro-Guerrero *et al*., 2016). It is assumed that common bean cultivation began as a weed along with maize, squash, and amaranth cultivated in cassava and sweet potato fields in Latin America (Purseglove, 1968). Throughout thousands of years, farmers grew mixtures of bean types that produced beans to meet their growing needs and taste preferences for their regions (Purseglove, 1968). Local landraces arose after the domestication period which led to the present morphological growth habits of determinant and indeterminate height, seed characteristics and size, and photoperiod length (Singh *et al*., 1991). Two wild gene pools of Mesoamerican and Andean common bean originally separated from a wild population over 100,000 years ago (Mamidi *et al*., 2013), which led to independent domestication events throughout Mexico and South America ~8000 years ago (Bitocchi *et al*., 2012; Bitocchi *et al*., 2013; Gepts *et al*., 1986; Mamidi *et al*., 2011).

Common bean provides people from developing countries a staple source of food and income. It does not require any industrial processing compared to other legume species such as soybean (*Glycine max*). Common bean provides 15% total daily calories and 36% of daily protein intake to humans when consumed (Schmutz *et al*., 2014) and is often referred to as "poor man's meat" (Noubissie *et al*., 2012). Health benefits of common bean include enhanced carbohydrates, iron, and fibers, which are cholesterol-free and therefore reduce the risk of chronic heart diseases and cancer (Geil & Anderson, 1994).

Worldwide bean production exceeds 26.8 million tons annually (FAOSTAT, 2018). Asia produces over 45%, the Americas 26%, and Africa 24% of the world's total yield of common bean (FAOSTAT, 2018) (Figure 1). Over half of the common bean production worldwide takes place

in low-income countries where food is scarce, and food security is critical for their survival (Porch *et al*., 2013). The other area of production includes countries like the U.S., where in 2016, nearly 1.8 million metric tons were produced on 1.63 million acres (NASS, 2017). Common bean alone contributes more economic value than other food legumes like peas, chickpeas, lentils and cowpeas underscoring the importance of its necessary production (Porch *et al*., 2013). Since common beans have a rich protein content, they are classified in the meats, fish, and eggs category of the food pyramid and sub-grouped in the vegetable group and are consumed regularly by both vegetarian and non-vegetarians.

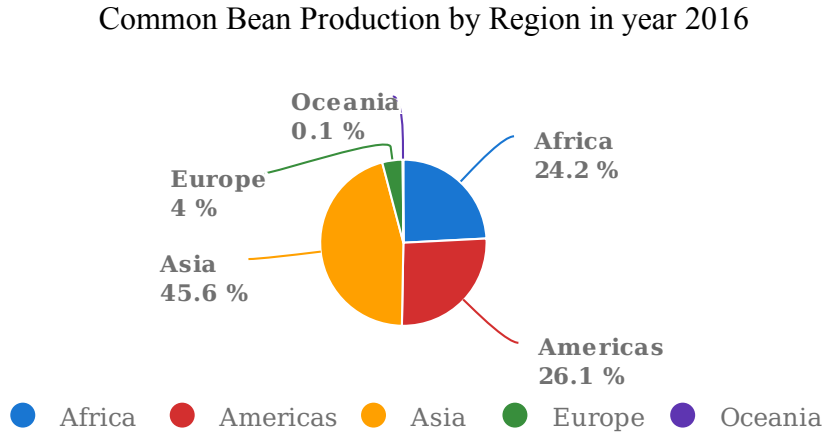

Figure 1: The top five common bean producing regions in the world for year 2016. Adapted from FAOSTAT (2018).

In addition to the health and nutritional benefits, common bean is a legume that is included in sustainable crop rotations for its ability to fix atmospheric nitrogen. A legume such as *P. vulgaris* forms a symbiotic relationship with *Rhizobium spp.* where these root nodule bacteria can convert dinitrogen (N_2) to a usable plant form of ammonia (NH_3^-) or ammonium (NH_4^+) (Mylona *et al*., 1995). This allows small scale farmers to minimize the use of costly fertilizer inputs that are normally needed for satisfactory crop yield, and provides an appropriate crop for reclamation on marginal land (Alexander, 1984).

Common bean belongs to the angiosperm phylum, characterized by a group of flowering plants that produces seeds within a carpel. It belongs to the leguminosae family defined by their flower structure, podded fruit, and their ability to form nodules with rhizobia (Faria *et al*., 1989). It is a dicotelydon seed, which produces two embryonic leaves upon emergence. These embryonic leaves supply the nutrients required for growth until the first true leaves are expanded. It is diploid with 22 chromosomes (2n=2x=22) and a self-pollinated crop with ~473 Mb of its ~587 Mb genome assembled (Schmutz *et al*., 2014). This crop is highly polymorphic with variations in its phenotype including growth habit, seed color, seed size, flower color, and size (Purseglove, 1968). Growth habits of common bean are highly variable ranging from tall vines reaching one to two meters tall indeterminate to determinate bush types. There are two major commercial classes of common bean-snap beans, and dry beans (Singh, 2001). This crop can be harvested for its fresh leaves, un-matured seeds and pods as snap beans, or harvested as a dry mature seed that can be stored for years under proper conditions. There are two gene pools, the Middle (Meso) American and Andean, with distinct characteristics (Mamidi *et al*., 2013). The Mesoamerican gene pool originated north of Peru into Columbia, Central America and Mexico and the Andean pool in southern Peru, Bolivia, and Argentina (Bitocchi *et al*. 2012). Common beans can be divided into different market classes and present a wide variety of seed coat colors from light colored pinto beans of the Meso American, to the dark red kidneys of the Andean gene pools. The typical market classes in the US from the Middle American gene pool include pinto, great northern, small red, and pink beans (Moghaddam *et al*., 2014). The market classes of the Andean gene pool include dark red kidney, white kidney, and cranberry (Mensack *et al*., 2010).

2.2 Abiotic and Biotic Stress

Common bean has been adaptable and suitable to different cropping systems, however, its production is often affected by many abiotic and biotic factors (Schwartz & Pastor-Corrales, 1989;

Dita *et al*., 2006; Wortmann *et al*., 1998). Due to its sessile nature, a plant exposed to stress responds by undergoing physiological changes that alter their growth (Pandey *et al*., 2017). Plant stress is defined as any factor that results in a molecular, physical and cellular responses activated by the detection of a stress factor by the plant. Abiotic stress is classified as a non-living factor including drought, heat, and salinity that hinders plant growth and productivity (Rabbani *et al*., 2003). Among the different abiotic stresses, salinity is one of the most severe limiting crop productivity (Yamaguchi & Blumwald, 2005; Shahbaz & Ashraf, 2013). Biotic stress is caused by infectious and transmissible organisms including fungi, bacteria, nematodes, viruses, and herbivores. Diseases affecting roots of bean plants are one of the most prevalent biotic stresses affecting common bean production.

2.2.1 Abiotic Stress

2.2.1.1 Salinity

The global food production will need to increase drastically to support an increase of the global population of 9.7 billion people in 2050 from the current population of 7.3 billion (United Nations, 2015). According to the FAO (2009), this 34% increase of the global population will require an increase of food production by 70%. Most arable land is already in production, which increasingly leaves the option to produce crops on marginal land especially in arid and semi-arid regions. However, these lower quality areas suffer from degradation, erosion, and salinity (Wild, 2003).

Salinity is a major abiotic factor that limits the production of important crops and threatens our food security. It has been estimated that 20% of the cultivated land worldwide, and 30% of the irrigated land suffers from high salinity issues (Jamil *et* al., 2011). Salinity concentration is comprised of dissolved minerals that are present in soil and water (Manchanda & Garg, 2008).

According to Richards (1969), soil is considered saline when the electrical conductivity of its saturation extract (EC_e) is more than 4 dSm^{-1}, or approximately 40 mM of NaCl (Shrivastava & Kumar, 2015). There are two main types of salinity that involve primary and secondary salinization. Primary salinity occurs naturally from natural processes (Manchanda & Garg, 2008), whereas secondary salinity occurs from human activities. The causes of primary salinity are the natural process of weathering parent rock material, and oceanic salt deposits carried inland. Sodium chloride (NaCl) is the most soluble and abundant form of salt that is released. Secondary soil salinity is caused by human activities such as fertilization, land degradation and using saline water for irrigation purposes (Bharti *et al*., 2012; Manchanda & Garg, 2008). When excess land clearing and irrigation provide more than enough water that is usable by the plants, it causes water tables to rise, thus mobilizing salts from the subsoil closer to the soil surface (Manchanda & Garg, 2008).

2.2.1.2 Impacts of Salinity on Crops

Salinity stress is known to cause problems within the plant at the molecular level, thus affecting physiological functions. It reduces the plants' ability to uptake and utilize water, which causes a reduction in metabolic processes and growth rate (Munns, 1993 & 2002). Cultivated crops respond to salinity exposed by showing restricted growth that results in an overall decreased yield (Shabaz & Ashraf, 2013; Yamaguci & Blumwalk, 2005). Roots affected by saline conditions lack the necessary root hairs that are important for the symbiotic relationship between the plant and the soil rhizobia to produce nodules. Reduced nodulation lowers the amount of necessary nitrogen necessary for survival and reproduction of legumes (Manchanda & Garg, 2008). Beans affected by salt stress appear stunted and chlorotic that can lead to necrotic tissue that reduces the surface area needed for the plant to photosynthesize. Soil salinity occurs in two phases: in the first phase

shoot growth begins to decrease once the soil salinity level reaches 40 mM; the second phase occurs when the salt accumulates to toxic levels in the leaves where they become necrotic (Bayeulo-Jimenez *et al*., 2012). During the osmotic phase, high concentrations in the soil surrounding the roots causes reduced water potential resulting in a water deficit that reduces the growth of young leaves (Munns & Tester, 2008). The first symptoms of a plant effected by salinity includes chlorosis, leaf drop, wilting, and root death (Johnson, 2000). The second or ion-specific phase is caused by phototoxicity where the sodium (Na^+) and chloride (Cl^-) ions accumulate in the tissues affecting the activity of cytosolic enzymes by disturbing the intracellular potassium homeostasis in plant cells (Marschner, 1995; Sairam & Srivastava 2002; Cuin & Shabala 2007; Chen *et al*., 2007). The accumulation of these ions occurs when they are translocated to the shoots and leaves through the xylem but they return to the roots through the phloem at a slower rate (Dikilitas & Karakas, 2014). Following these two phases, leaf senescence of mature leaves occurs and the loss of photosynthetic leaf surface decreases resulting in yield loss.

The essential cellular functions that rely on potassium (K^+) are limited due to the competition from Na^+ for binding sites. It has been noted that plants have developed adaptive mechanisms to tolerate salt stress through enhanced enzymatic and physiologic reactions and phenol secretion (Abdi *et al*., 2015). Salt tolerant genotypes are known to produce higher levels of antioxidant enzymes as one of the mechanisms for salt tolerance compared to the more sensitive cultivars (Logan, 2005). Exposure of plants to salinity stress results in the production of a byproduct called reactive oxygen species (ROS) that injures the cellular components of the plant (Mittler, 2002). Formulations of the ROS include superoxide, hydrogen peroxide, and hydroxyl radicals that weaken the membrane, and DNA modifications that lead to structural abnormalities and eventually cell death (Mittler, 2002). There are no current statistics on the amount of yield and production losses of common bean due to salinity. However, common bean is reported to be a very

sensitive crop suffering yield losses at soil salinity levels less than 2 dS m^{-1}, which is half the rate that soil is considered saline at (Läuchli, 1984). A previous study by El-Abyad *et al*. (1992) evaluated 11 isolates of *Fusarium* spp. and *Rhizoctonia solani* from sugarbeet under saline conditions where the mycelial growth was increased in most of the isolates.

2.2.2 Biotic Stress

2.2.2.1 Fungal Pathogens

Aside from abiotic stresses, agricultural yield losses occur from biotic factors including pathogens (Alvarez, 2004). Several staple crops including rice, wheat, beans, and corn are affected annually by pathogens (Oerke, 2006) that jeopardize our food security. Diseases caused by fungi, bacteria, and viruses are major constraints to bean production. Foliar diseases caused by biotrophic fungal pathogens including rusts, downy mildews, and powdery mildews often limit bean production (Sillero *et al*., 2006) in addition to several bacterial and viral diseases. In order for disease to occur, there are three components that must be present including a susceptible host, virulent pathogen, and ideal environmental conditions. Root diseases are known to be more severe when abiotic stresses, such as temperature, drought, water logging and salinity, are present (Thung & Rao, 1999). These conditions can exacerbate pathogen infection. Worldwide, grain legumes including beans are produced as a major food crop however, they have limited genetic resistance to several economically important pathogens (Shafique *et al*., 2014). The pathogens *R. solani* and *F. solani* are part of the root rot disease complex that causes damping off and seedling blight resulting in reduced plant stand, nitrogen fixation, and root vigor (Gossen *et al*., 2016). When the roots become affected, and begin to rot, the plant loses its ability to uptake water and nutrients due to the colonization of the fungi within the xylem causing wilting and plant death (Shafique *et al*., 2014). These pathogens ultimately reduce root quality and thereby affect yield (Henriquez *et al*.,

2014). Yield losses of up to 100% in susceptible cultivars have been reported (Nzungize *et al*., 2012; Mukankusi *et al*., 2011) especially in combination with abiotic stresses (Harveson *et al*., 2005).

The filamentous fungal genera *Fusarium* is anticipated to be the more serious root invading pathogen (Kraft & Pfleger, 2001) and *Fusarium* root rot (FRR) is one of the most destructive diseases of beans throughout the world (Abawi & Corrales, 1990; Macedo *et al*., 2017). The FRR disease causes yield losses up to 84% (Park & Tu, 1994) in numerous countries throughout the world (Nzungize *et al*., 2012). Other pathogens causing root rots are other *Fusarium* spp., *Phythium* spp., and *Rhizoctonia solani* (Abeysinghe, 2007). Pathogens such as *F. solani* (Mart.) Sacc. f. sp. *phaseoli* (Burkholder) W.C. Snyder & H.N. Hans (Burke & Hall, 1991) and *R. solani* (Kühn) (Valentín Torres & Vargas, 2016) are soil inhabiting fungi that cause root rots.

Fusarium root rots are favored by soil compaction that restrict root growth and early cool/wet growing parameters that affect beans as early as planting to V3 stage. However, later in the growing season, additional abiotic and biotic factors, can increase the disease severity and yield can be drastically reduced in upwards of 86% (Cichy *et al*., 2007; Abawi & Corrales, 1990). Leaf senescence follows and inhibits the plants' ability to photosynthesize resulting in reduced pods, seed size and yield. Some of the abiotic stresses that intensify root rots include deep planting, compaction (Burke, 1965; Miller & Burke, 1985), cool temperatures, high or low pH, low fertility, pesticide and fertilizer injury, flooding, and drought (Burke *et al*., 1969; 1972; Miller & Burke, 1985; 1977). The disease is first observed as long red to brown longitudinal streaks on the main taproot and hypocotyl. The initial red lesions will turn dark and necrotic. The taproot later turns dark brown and cracks which leads to shriveling and dying. Clusters of fibrous adventitious roots can be seen extending out above the taproot. As the disease progresses, it expands to the whole underground root system and into the pith of the stem. The entire root system begins to die showing

chlorotic and necrotic foliage that reduces the plants' photosynthetic area. Infected plants exhibit symptoms of stunted growth, pale leaf color, and grow more slowly than healthy plants leaving behind un-even plant stands (Schwartz & Pastor-Corrales, 1989; Abawi & Pastor-Corrales, 1990; Saettler & Hall, 1991; Abawi *et al*., 2006).

The pathogen *F. solani* is the asexual (anamorph) phase, which produces macroconidia and its equivalent sexual (teleomorph) phase is *Nectria haematococca,* which produces ascospores (Abawi *et al*., 1985; Abawi, & Pastor-Corrales 1990). Overwintering survival structures are in the form of thick walled chlamydospores that colonize plant residues, and which can stay dormant and persist in the soil for as long as 30 years (Schwartz *et al*., 2005) or until the conditions are favorable for germination (Schwartz & Corrales, 1989; Tseng *et al*., 1995; Schwartz *et al*., 2005). When the pathogen's spores are in the soil, they are controlled by fungistasis. Fungistatis can occur in most soils where the fungal propagules are restricted from germinating and growing (Garbeva *et al*., 2011) that was first described by Dobbs and Hinson (1953) as the event of the inhibition of germination of fungal spores or the growth of hyphae in soils. When the fungistasis is reversed by the nutrient exudates from germinating seed and root tips, the spores germinate and directly penetrate the bean root tissue, through wounds or natural openings (Abawi, 1980; Hall, 1991). When plant tissues become necrotic and die, the conidia and hyphae transform into the thick walled chlamydospores to survive until the next host is available (Schwartz *et al*., 2005). Pathogen dissemination occurs by humans, rain, wind, animals, farm machinery, and contaminated seed (Abawi & Corrales, 1990). The pathogen has been shown to germinate and reproduce near non-host species without causing disease therefore increasing or maintaining its population in the absence of beans (Schwartz *et al*., 2005). Management strategies to reduce bean root rot include planting in well-drained soils with little compaction, minimizing plant stress, and following integrated pest management approaches.

Rhizoctonia species are often associated with root rot diseases in some of the most economically important crops. The fungal pathogen, *R. solani* (teleomorph=*Thanatephorus cucumris* Frank (Donk)), is a member of the Basidiomycota phylum and is a filamentous fungus that currently is not known to produce asexual spores, however, produces sclerotia that survive in the soil as a survival structure. This pathogen has a wide host range and is distributed throughout the world. This pathogen causes significant yield reductions globally of many important staple crops (Sturrock *et al*., 2015). It has been discovered across many geographical regions providing evidence of the diverse and widespread features of this pathogen (Galindo *et al*, 1982; Ohkura *et al*, 2009; Pastor-Corrales, & Abawi, 1988). This pathogen is a facultative parasite that can survive in the soil as a saprophyte (Ogoshi, 1996), which reduces yield of common beans between 42-88% (Beebe *et al.,* 1981; Tan and Tu, 1995). The pathogen can be spread by humans, rain, wind, animals, and machinery.

To date, the *R. solani* isolates have been classified into 14 anastomosis groups (AG) based on their hyphal fusion, morphology, pathogenicity, and DNA homology (Carling *et al*., 2002). Isolates within AG groups may prefer similar hosts and show similar symptoms (Dorrance *et al*., 2003). Isolates responsible for root and hypocotyl rot of bean belong to the AG2-2 and AG4 groups (Valentín Torres *et al*., 2016) that produce a white velvet appearance and favor temperatures around 28°C (Sherwood, 1969).

The pathogen *R. solani* is a major culprit causing destructive root rots and damping-off worldwide affecting many species of *Phaseolus* beans including snap, green, lima, and dry beans. *R. solani* prefers high soil moisture and warm temperatures in addition to high organic matter content. Seeds that have delayed emergence or are stressed have an increased chance of infection. Although the pathogen does not produce asexual spores or conidia, it will sometimes produce sexual spores or basidiospores. The fungus can survive in the soil for many years as mycelial

threads inside decaying organic matter or as sclerotial structures. These structures are long term survival structures containing hyphae that live dormant in the soil for years until the conditions are favorable. Hyphae develop under favorable conditions during these conditions and will then penetrate a host using an appresorium and infection cushion. Attraction of the fungus to the host is initiated by chemical exudates released by growing plant cells or decomposing plant debris. Once in contact with the host, hyphae branching and the formation of infection structures of swollen hyphae or appresoria occurs. (García *et al*., 2006). From the hyphal branches, a specialized structure called the infection cushion extends into an infection peg that penetrates the cuticle and epidermis (Keijer, 1996). Fungal hyphae penetrate the host inter and intra-cellularly using an appresorium and secrete several hydrolytic enzymes that damage the plant cell wall and aid in pathogen entry (García *et al*., 2006). The inoculum then waits as dormant mycelial threads or sclerotia until a new substrate becomes available and the cycle is initiated again. Management practices such as tillage, crop rotation with grains, planting in warmer drier soil for faster emergence, and drainage can improve the risk of *R. solani* infection (Secor & Gudmestad, 1999).

2.2.2.2 Salinity on Fungal Growth

Environmental factors such as salinity that pose abiotic stresses may have effects on the virulence, growth, and reproduction of fungi (Howell & Erwin, 1995; Dikilitas, 2003; Roos *et al*., 2011). Environmental factors especially salinity, occur in arid and semi-arid regions of the world. Pathogens that are exposed to these factors could possibly change their behavior and adapt to adverse conditions. High salt concentrations are thought to lower fungal mycelial growth and conidia formation through the toxic effects of salinity (Jones *et al*., 2011; Egamberdieva, 2012). The response of fungi to the effects of salinity are similar to the way crops respond (Mahmoud *et al*., 2007). Using NaCl as soil amendments have been proposed throughout the years to reduce

root disease severities (Engel & Grey, 1991; El-Mougy & Adbel-Kadar, 2009; Elmer, 1992). It is believed that pathogens that have their modes of reproduction and development reduced by salinity will over time have the potential to adapt (Dikilitas & Karakas, 2014). Using NaCl as a control method may favor development of salinity resistant pathogens much like the fungicide resistant pathogens that we currently face. Aside from negative effects, salinity can also promote fungal growth. In some studies, pathogens have been shown to respond to saline conditions by increasing growth and increasing their pathogenicity by enzymatic and cellular metabolic activities (Dikilitas & Karakas, 2014). When under salinity stress, *R. solani* was found to be more virulent in causing disease on sugar beet than *Sclerotium rolfsii* (El-Abyad *et al*., 1992). *Botrytis cinerea* the causal agent of grey mold on tomato had a significant growth increase response to NaCl up to a concentration of 300 ppm, and an increase in spore production and germination (Boualem *et al*., 2015). Different pathogens have been seen to respond differently to salinity posing a potential problem in predicting the effect of increasing salinity on disease without pathogen specific studies in the future.

2.3 Molecular Pathogenicity and Disease Resistance

Common bean has 473 Mb of its 587 Mb genome assembled (Schmutz *et al*., 2014). Bean root rot is one of the most important diseases in terms of the reduction in plant growth and yield. Most commercial common bean cultivars are susceptible to the pathogens that cause bean root rot. Plant pathogen interactions have well understood mechanisms that utilize the activation of signals and responses against an attack to further prevent disease infection (Gururani *et al*., 2010). These specialized mechanisms respond by the recognition of pathogen effectors through host R-genes (Belkhadir *et al*., 2004). Plant resistance (R) genes have been used efficiently in crop research for crop improvement programs for developing resistant cultivars that minimize pathogen growth and

limit damage to the plant (Gururani *et al*., 2010).

2.3.1 Plant Disease

Plant disease occurs under the compatibility of a host and a pathogen. For a disease to occur, a host, pathogen, and suitable environment must be present. When the conditions for the disease are favorable, disease occurs and the plant must rely on internal mechanisms to protect against pathogen entry. When the pathogen is successful in infection, it relies on its nutritional needs to survive, whether it is killing the plant and feeding from it, or keeping the plant alive in order to survive. Before a pathogen has a successful infection, plants deploy responses to disease attacks using two types of disease resistance, basal defense and R-mediated defense.

2.3.2 Basal Defense

Basal defense or innate immunity includes the non-host and host resistance that is the first line of defense against pathogens. Plants are sessile and cannot protect themselves other than natural barriers or internal mechanisms. The network of pattern recognition and plant immunity through the 'zig zag' model was described by Jones and Dangl (2006) to show how the plant immunity response is triggered by the pathogen. The use of pattern recognition signals to react to the pathogen-associated molecular patterns (PAMPs) or often referred to microbe-associated molecular patterns (MAMPs) (Jones & Dangl, 2006; Garzón *et al*., 2013). P/MAMPs are important functional machineries of pathogens that allow the host plant to recognize itself from the pathogen and signal to the innate immunity (Tang *et al*., 2012). Plant disease resistance is categorized by host resistance or non-host resistance. Host resistance is the specific resistance to a cultivar or genotype, whereas non-host resistance is the most common and carries resistance across all members of a plant species (Heath, 2000). Non-host resistance also occurs when the conditions are not suitable enough for infection or for the pathogen to survive (Hammond-Kosack, & Jones,

1996). There are two types of non-host resistance, namely Type 1 and Type 2. Type 1 is the most common type of non-host resistance where there is no hypersensitive response (HR) and usually no visible symptoms (Uma *et al*., 2011). In Type 2 non-host resistance, a programmed cell death HR induces necrosis by releasing detoxifying enzymes to prevent the spread of the pathogen within the plant cell tissues (Uma *et al*., 2011). However, pathogens have developed ways to suppress the basal defense mechanisms and in response plants trigger their HR. When the HR defense is triggered, the necrotic tissue does not provide the pathogen the resources to survive and reproduce. After may studies of the (*R*) and (*Avr*) protein interactions (Jia *et al*., 2000; Tang *et al*., 1996), it remained unverifiable leading to the 'guard hypothesis' (Van Der Biezen & Jones, 1998; Jones & Dangl, 2006). Resistance is activated when the (*R*) gene proteins interact with a plant guard protein. This protein has been modified by the attacking pathogen to create the ideal environment or a product of the pathogen attack (Shen *et al*., 2002).

2.3.3 R-gene Mediated Pathogen Resistance

When pathogens attack a plant, they produce molecules called effectors that are encoded by avirulence (Avr) genes that are released directly into the host tissue during infection. These effectors can either change the condition of the plant to benefit the attacking pathogen, or alter the plant defenses (Collmer, 1998; Hammond-Kosack & Kanyuka, 2007). However, plants have developed an immune response using the (R)gene mediated pathogen resistance (Nimchuk et al., 2003). H.H. Flor (Flor, 1956) studied the interaction between flax and the flax rust pathogen. His studies led to the gene-for-gene theory by finding that plants and pathogens inherit resistance and avirulence in gene pairs. For every (R) gene within the host, the pathogen has a complementary (Avr) gene where these two genes interact. If one of the genes is missing, there will be no gene-for-gene resistance and under some circumstances where the pathogen does not carry the (Avr)

gene, disease will still occur. These (R) genes are of several types with the most common type consisting of Leucine Rich Repeats (LRR), Nucleotide Binding Site (NBS), and Toll/Interleukin-Receptor (TIR). LRR plays an important role in the pathogen recognition (Takahashi, 1985). The presence of the NBS region in the (R) gene indicates nucleotide triphosphate binging is needed for protein function that is an essential piece to disease resistance (Bent, 1996). NBS-LRR domains can be additionally organized into subdivisions of families that include the TIR and non TIR (Toll/Intereukin-1 Receptor) domains (Meyers et al., 1999). The Toll receptors have a role in the innate immune response of the first line basal defense where it has been shown to be abundant in Arabidopsis (Jebanathirajah et al., 2002).

2.3.4 Challenges of Disease Resistance and Future Outlooks

Conventional breeding for resistance is a long-term process that uses back crossing to gain an introgression of a resistance gene from one gene pool to another that requires many generations of hybrid breeding before back crossing can even begin (Gurarani *et al*., 2012). Integrating more than one (*R*) gene is problematic and time consuming because of the epistatic interfaces between (*R*) genes that requires testing with many strains of the pathogen (Mezadi *et al*., 2016). Pyramiding genes in a single cultivar used for durable resistance can be carried out using marker assisted selection (MAS) (Collard & Mackill, 2008). For more than one hundred years, the use of conventional methods to try to manipulate the genes used as immune receptors in plants have been carried out (Nishumura *et al*., 2015). Modern genomic and bioinformatics tools and techniques have increased the use of expressed sequence tag (EST) approaches by providing genome-wide analyses, transcriptomes, and gene expression profiles (Wibberg *et al*., 2014). Plant-pathogen interactions have also been studied to understand the relationships and mechanisms behind it by using ESTs, whole genome sequences, and gene expression data (Gurarani *et al*., 2012). A

transcriptome analysis of *R. solani* described a first glance into predicted functions of the interaction of the fungal pathogen and the plant (Wibberg *et al*., 2014). Methods of using cloned resistance and effector genes to provide acquired resistance provides localized defense responses associated with HR; the most effective mechanism used to minimize an attack. Research into the plant-pathogen interaction to validate *Avr-R* gene combinations, where Rushton *et al*. (2002) proposed combining (*Avr*) and (*R*) genes within a plant genotype to 'trigger' HR. During the pre-genomics era, molecular markers linked to genes of interest (GOI) was based on the development of genetic maps (Michelmore *et al*., 1991) whereas the post-genomics era offers the complete genome sequence of plants such as common bean offering rapid development of markers linked to (*R*) genes (Meziadi *et al*., 2016). Future genomics studies in common bean will improve breeding programs, specifically the disease resistance and improving common bean performance worldwide.

CHAPTER III: MATERIALS & METHODS

3.1 Evaluating the Effect of Increasing Salinity on Fungal Pathogens

The effect of increasing salinity on fungal pathogens was measured in terms of radial mycelial growth, fungal biomass, spore germination and disease severity evaluation.

3.1.1 Radial Mycelial Growth

In this study, two pathogens commonly associated with common bean root rots were used. These pathogens were *Fusarium solani* f. sp. *phaseoli* and *Rhizoctonia solani*. There were three isolates of *R. solani* and one isolate of *F. solani* (Table 1). These isolates were isolated from common bean plants in Michigan, Nebraska, and Delaware where they are common isolates that are used for screening purposes. The *R. solani* isolates belong to the anastomosis groups AG2-2, and AG4.

Table 1: Pathogens and isolates used in this study.

Pathogen	Isolate
Fusarium solani	MI-MIC-B8
Rhizoctonia solani	UD8
	WN11
	WN293

Fresh plates were started from a preserved glycerol stock culture in glycerol kept at -80° C. The cultures were grown on potato dextrose agar (PDA) (Difco, Sparks, MD) for seven days until sub-culture. At day seven, a 5 mm plug was obtained from the growing edge of the colony on the original plate. It was then placed on a fresh PDA plate and allowed to grow for one week before they were used for initiation of plates amended with NaCl or further sub-cultured for use in future experiments. Media for this study was made using PDA that was amended with NaCl using

four concentrations and a control (Table 2). The media was prepared by making 1.5% PDA (24 g/L) and adding corresponding amounts of NaCl and then autoclaved at 121°C for 15 minutes. Once the sub-cultured plate was seven days old, using a cork borer a 5mm plug was excised and placed in the center of the Petri dish containing media with the different NaCl concentrations. Each of the concentrations had three biological treatment replications. The Petri dishes were sealed with Parafilm and placed upside down in a Percival growth chamber (Percival Scientific, Perry, Iowa) at 24°C under 65% ± 1 RH. Growth of the mycelium was recorded at day three, six, and ten by taking perpendicular growth measurements in millimeters across each plate. The experiment was repeated three times for each pathogen and isolate.

Table 2: Concentrations and amount of NaCl added to make the NaCl-amended PDA media.

Concentration of NaCl (mM)	Amount of NaCl Added (g)	Amount of Agar Added (g)	Total Volume (mL)
0	0.0	2.625	175
50	0.5118	2.625	175
150	1.5356	2.625	175
200	2.0475	2.625	175
250	2.5593	2.625	175
400	4.0950	2.625	175

3.1.2 Fungal Biomass

All the isolates of *F. solani* f. sp. *phaseoli* and *R. solani* used in the mycelial growth study were also used for assessment of fungal biomass. The procedures for starting fresh cultures on PDA media that were used for starting liquid cultures were also same as the mycelial growth study. Media for the fungal biomass study was prepared using potato dextrose broth (PDB) (Difco, Sparks, MD) (24 g/L) and dH_2O that was autoclaved at 121°C for 15 minutes. A separate 1M

solution of NaCl (Fisher Scientific, Hampton, NH) was made using dH_2O and granular NaCl (58.54 g/mol) and autoclaved at 121°C for 15 minutes. The respective amounts of PDB and volume of 1M NaCl were measured and combined in 250 mL Erlenmeyer flasks for a total volume of 50 mL (Table 3). From the seven-day old culture plates, using a cork borer six 5 mm plugs were excised radially from the center of the plate outward to capture all growth stages of the mycelium and were added to each flask. Each flask containing the respective concentrations of NaCl broth and plugs had three biological replications. The twelve flasks were placed on an orbital shaker (Thermo Scientific MaxQ2000) in a Latin Square Design at 150 rpms for fourteen days (Figure 2). The orbital shaker was placed in a Percival growth chamber (Percival Scientific, Perry, Iowa) at 24°C under 65% ± RH. Once the fungus grew for fourteen days, it was harvested by filtering through Whatman #1 filter paper (GE Healthcare, UK), allowed to air dry for three days, and weighed on a balance (Mettler Toledo Columbus, Ohio) in grams and converted to milligrams. The study process was repeated three times for each pathogen and isolate.

Table 3: Amount of PD broth and 1M of NaCl added to the flask.

Concentration of NaCl (mM)	Amount of PDB Added (mL)	Amount of 1M NaCl Added to PDB (mL)	Total Volume (mL)
0	0.0	0	50
50	47.5	2.5	50
150	42.5	7.5	50
400	30	20	50

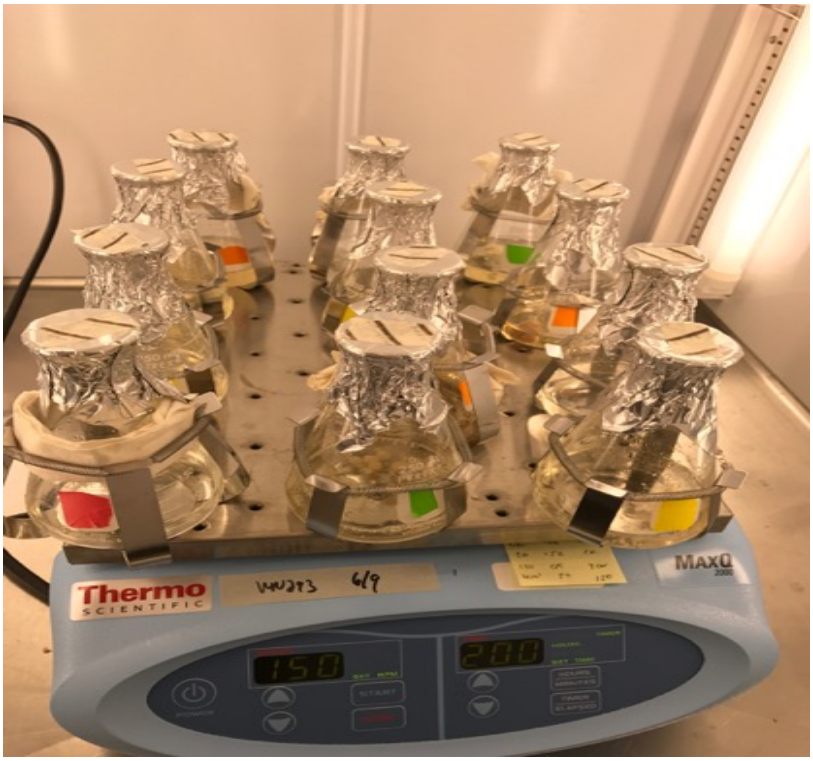

Figure 2: Flasks containing 5 mm mycelia plugs and sodium chloride (NaCl) amended potato dextrose broth (PDB). Orange=0 mM, green= 50 mM, yellow= 150 mM, and red= 400 mM. Flasks rotated @ 150 rpms on an orbital shaker until day 14 when the biomass was harvested.

3.1.3 Spore Germination

Spore germination was assessed using the quantity of germinating spores under increasing NaCl concentrations. Only *F. solani* was used in this study since it produces spores asexually (Figure 3). Fresh plates were started from long term glycerol stock from the -80 °C freezer using the same procedures as described in the mycelial growth experiment. Seven days after a fresh plate was started, a 5mm plug was excised and placed on 1.5% PDA to grow for fourteen days to allow adequate spore production. At fourteen days, 5 mL of sterile dH_2O was added to the plate and was scraped with an inoculating loop to release the spores. The spore solution was pipetted into a 10

mL snap cap tube and an additional 5 mL of dH_2O was used to wash the plate again and added to the tube. The spore solution was vortexed to make the solution homogenous and an aliquot of 100 µL was added to a series of 10^{-1}, 10^{-2}, and 10^{-3} dilutions in 1.5 mL tubes that contained 900 µL of sterile dH_2O. Each dilution aliquot was observed under the microscope using a haemocytometer where the 10^{-3} dilution had a countable number of spores. From the 10^{-3} aliquot, 10 µL was inserted into the haemocytometer where measurements of each square were taken and averages were calculated to have a final spore count of 400 spores/mL. Calculations and dilutions were made to pipette 40 µL of the spore solution onto 2% water agar (WA) amended with NaCl to have a final spore count of 40 spores per plate for each concentration once the suspension was uniformly spread with a spreader. Each treatment consisted of three biological replications. The WA was made using dH_2O with a respective amount of added sodium chloride and autoclaved at 121°C for 15 minutes (Table 4). Plates were sealed with Parafilm and placed in the Percival growth chamber (Percival Scientific, Perry, Iowa) at 24°C under 65% ± RH. Germinating spores were counted for each plate for all the treatment concentrations after three days. This study was repeated three times.

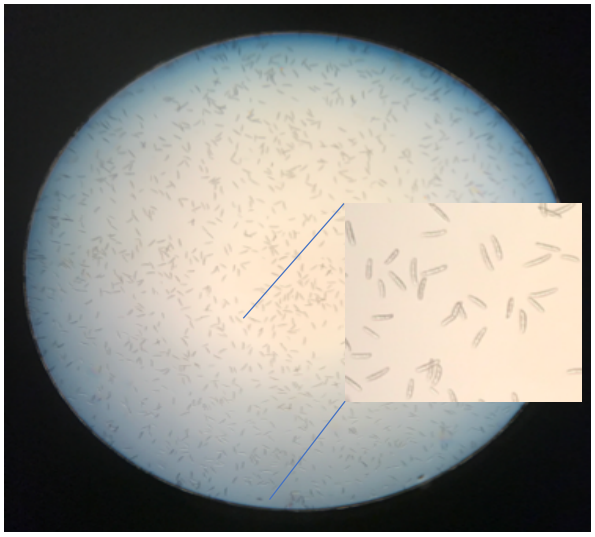

Figure 3: Microscopic view of *F. solani* macroconidia at 14 days old viewed under 40x magnification. Conidia have 3 to 4 septa on average and a curved boat shape.

Table 4: Amount of NaCl added to the 2% water agar used for spore germination test

Concentration of NaCl (mM)	Amount of NaCl Added (g)	Amount of Agar Added (g)	Total Volume (mL)
0	0.0	3.5	175
50	0.5118	3.5	175
150	1.5356	3.5	175
200	2.0475	3.5	175
250	2.5593	3.5	175
400	4.0950	3.5	175

3.1.4 Disease Evaluation

Growth chamber experiments were performed to determine the influence of increasing salinity on root rot severity. In this study, one isolate of each of the two pathogens *R. solani* (WN293) and *F. solani* f. sp. *phaseoli* (Mi-MIC-B8) were used to inoculate common bean roots using a modified paper towel and sand corn meal method (Bilgi *et al*., 2008). Using the same procedures of starting fresh cultures from the glycerol stock in the -80°C freezer as described in the initial mycelial growth study, fresh plates were started and grown for seven days. After the culture had grown for seven days, a 5mm plug was taken from the original plate and placed on a fresh PDA where this plate was used to excise plugs for making the inoculum. A sand corn meal medium mixture using the ratio of 5 g sand and 45 g cornmeal (w:w) was autoclaved at 121°C for 20 minutes. Using 250 mL Erlenmeyer flasks, the medium was filled to the 50 mL line of the flask or 100 g and 10 mL of sterile dH_2O was added. Using a cork borer, sixteen 5 mm plugs were excised and added to the sand corn meal medium and mixed thoroughly by stirring with a sterile glass rod. Flasks were placed in the Percival growth chamber at 24°C under 65% ± 1 RH for seven days and stirred daily for uniformity of pathogen growth until it was used for inoculation of bean roots.

Seeds of the common bean cultivar 'Redhawk', were surface sterilized in 5% household bleach followed by a triple rinse in sterile dH_2O. The seeds were then planted in a sterile growing media containing Promix (Premier Tech Horticulture, Quarkertown, PA) and vermiculite (Vigoro) in a 1:3 ratio (w:w). Pots containing the seeds were placed in a Conviron growth chamber using 16/8hr (day/night) cycle at 24°C under 55% RH ±5. Once the plants reached ten days after planting (DAP) and the first two true leaves had opened, they were removed from the pots and washed carefully with tap water. Two sterile paper towels were placed on the counter and a root was placed on the towel. The sand corn meal inoculum prepared was spread over the root at 5g/root and two

more layers of towels were used to cover the root. The root of each plant was then rolled up with the bottom folded to prevent inoculum loss and placed in a 50 mL conical tube (CORNING Corning, NY). Roots were placed in Styrofoam tube holders and placed in a Conviron growth chamber using 16/8hr (day/night) cycle at 24°C under 55% RH ±5 until disease severity ratings were recorded 10 days after inoculation (DAI) (Figure 4). Roots were watered with 10 mL of a NaCl solution using a modified version of the gradual step salt acclimatization procedure to prevent osmotic shock (Sanchez *et al*., 2008).

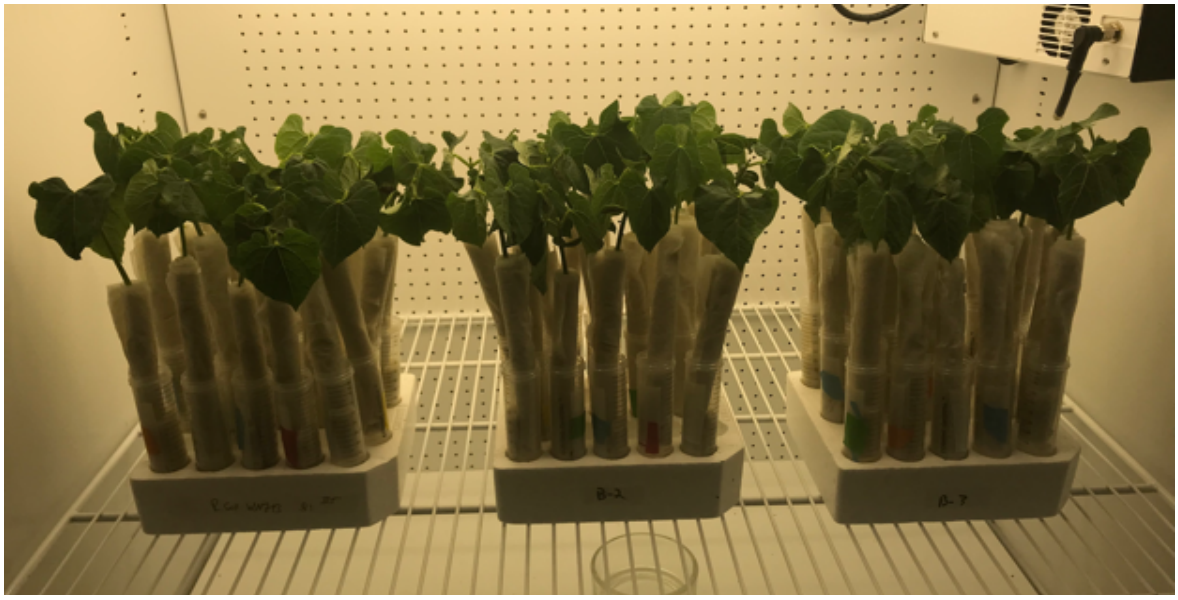

Figure 4: Common bean plants that were inoculated and placed in a growth chamber 10 DAP. Plants were watered with respective NaCl amounts until 10 DAI when roots were evaluated for disease.

On inoculation day, and the following three days bean plants other than the controls received 10 mL of 50 mM NaCl water to reduce osmotic shock. Four days after inoculation the concentration increased by 50 mM until final concentrations of 50, 100, 150, and 200 mM were reached. For each treatment, there were three biological replicate bean plants (Figure 5). Ten days after inoculation, roots were removed and washed with tap water to remove inoculum. Roots were rated for disease severity based on a 1-to -5 scale, where 1 = healthy no symptoms, normal root

development; 2= tissue discoloration without necrosis; 3= lesions with extensive tissue discoloration; 4= nearly complete root necrosis restricted root length; 5= complete root rot, restricted root length rotted based on lesions loss of visual root mass, and root discoloration (Muyolo *et al*., 1993; Hagerty *et al*., 2015). Root rating numbers were transformed using R* = (R1.5 – 1)/1.5 to obtain a linear scale as described by Krause *et al*. (2001). The experiment was repeated three times for each pathogen.

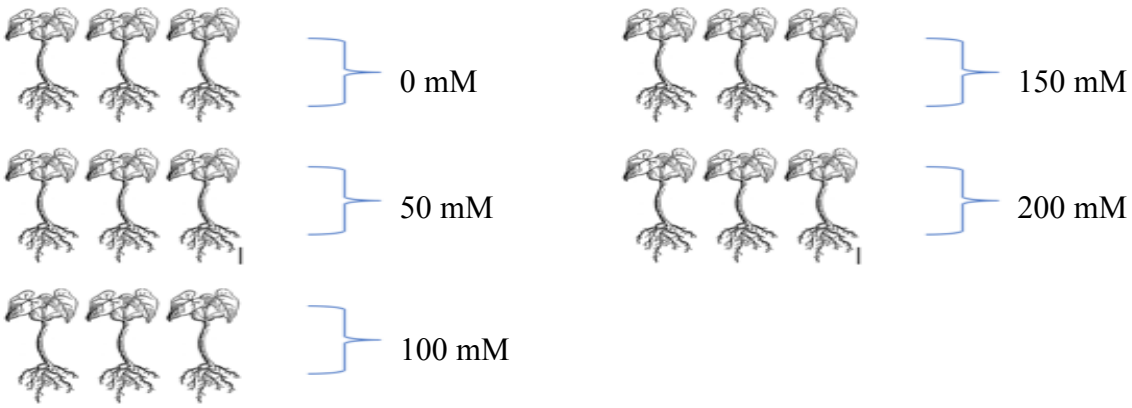

Figure 5: Schematic of bean roots and replicates used for each treatment concentration.

3.2 Statistical Analysis

Statistical analyses for the above studies was performed using R statistical software (version 3.2.2, R Core Team 2013) using the package 'agricolae'. Homogeneity of samples between experiment numbers were compared using LEVENE's test to compare variances. If no significant differences were detected between repeated experiments the data was combined using sample replications of each treatment and analyzed using a one-way ANOVA. Using Fisher's protected least significant difference (LSD) test, means from significant tests were separated.

CHAPTER IV: RESULTS

Data from all three repeated experiments for each of the studies were combined and analyzed after determining there were no significant differences between replicated experiments. Using a one-way ANOVA, significant differences of the means were determined followed by a mean separation using Fisher's protected least significant difference (LSD) test.

4.1 Mycelial Growth

The isolates of each pathogen had a variation of growth among the concentrations of NaCl and the day of measurement. The effect of NaCl on mycelial growth was studied using solid media. The growth of the *F. solani* isolate at day 3 (Figure 6), showed significant differences compared to the no NaCl (control) for all concentration at and above 150 mM (Table 5).

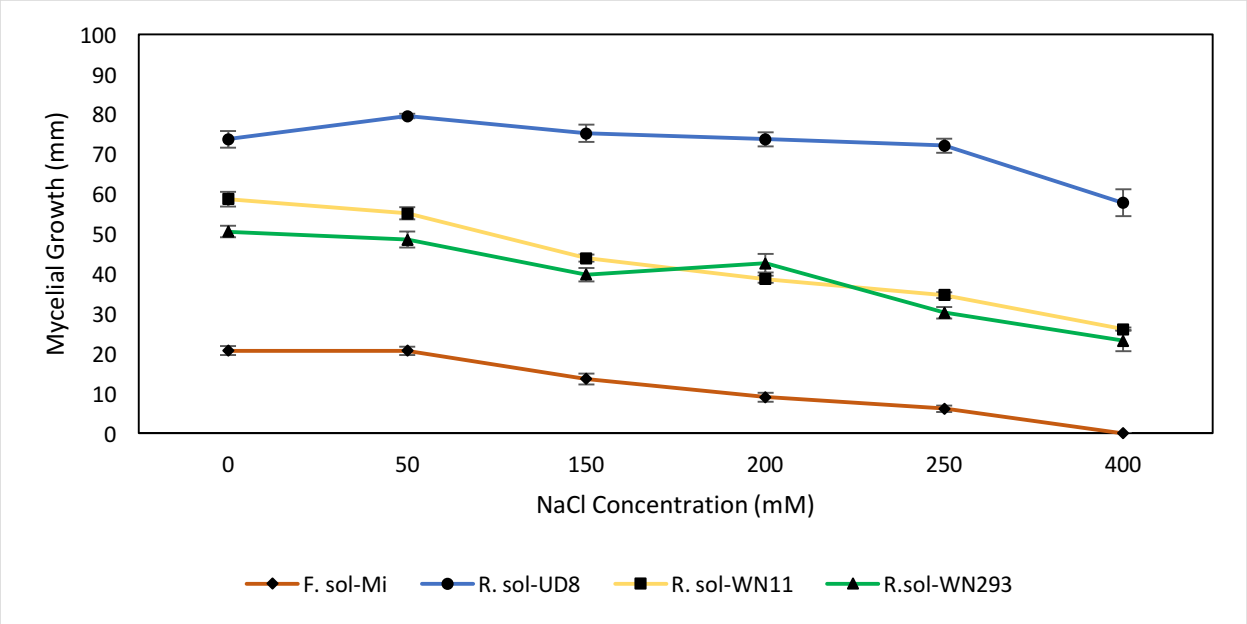

Figure 6: Effect of sodium chloride (NaCl) on the radial mycelial growth measured at day 3 of *F. solani* and *R. solani* isolates that are commonly associated with root rot of common bean. *Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test). Error bars represent standard error of the mean.

Table 5: Salinity effect on mycelial growth at various millimolar concentrations of sodium

chloride (NaCl) at day 3

Pathogen-Isolate	0	50	150	200	250	400
F. solani -Mi	20.72^{a}	20.66^{a}	13.61^{b}	9.05^{c}	6.16^{d}	0^{e}
R. solani- UD8	73.66^{ab}	79.44^{a}	75.16^{ab}	73.72^{ab}	72.05^{b}	57.78^{c}
R. solani- WN11	58.66^{a}	55.11^{b}	43.88^{c}	38.66^{d}	34.66^{e}	26.11^{f}
R.solani-WN293	50.55^{a}	48.55^{a}	39.77^{b}	42.61^{b}	30.22^{c}	23.22^{d}

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).

On the other hand, *R. solani* isolates showed a lot of variation in their sensitivity to NaCl. The isolate UD8 only had a significant growth difference with the control at the 400 mM concentration, the isolate WN293 showed difference only at 250 and 400 mM and the isolate WN11 had significant growth difference with the control at all the NaCl concentrations. At day 6 of growth under salt stress (Figure 7), *F. solani* followed the same trend of significance as in day 3. The *R. solani* UD8 had maximum growth possible and reached the edges of the plate and was not significantly different from the control. The isolate WN293 was significantly different from the control at concentrations of 150, 250, and 400 mM and the isolate WN11 were significantly different at all concentrations compared to the control (Table 6).

Mycelial growth observed at day 10 (Figure 8) for *F. solani* isolate and *R. solani* isolate WN11 demonstrated significant differences as compared to the control for concentrations at 150 mM and above. The other *R. solani* isolate WN293 showed significant differences with the control only at 250 mM and 400mM NaCl concentrations. (Table 7). The overall growth of the pathogens was reduced throughout the increasing concentrations except for the UD8 isolate of *R. solani*.

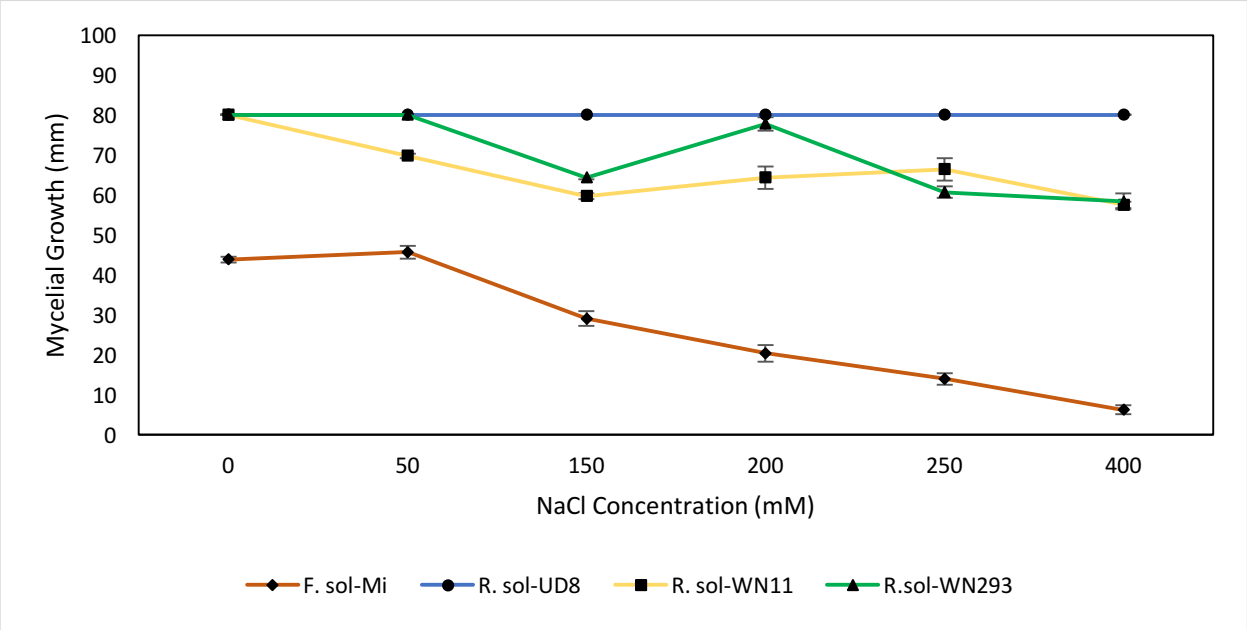

Figure 7: Effect of sodium chloride (NaCl) on the radial mycelial growth measured at day 6 of *F. solani* and *R. solani* isolates that are commonly associated with root rot of common bean. *Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test). Error bars represent standard error of the mean.

Table 6: Salinity effect on mycelial growth at various millimolar concentrations of sodium chloride (NaCl) at day 6

	NaCl Concentrations (mM)					
Pathogen-Isolate	0	50	150	200	250	400
F. solani -Mi	43.83^{a}	45.66^{a}	29.05^{b}	20.38^{c}	13.94^{d}	6.22^{e}
R. solani- UD8	80.00	80.00	80.00	80.00	80.00	80.00
R. solani- WN11	80.00^{a}	69.77^{b}	59.66^{de}	64.33^{cd}	66.44^{bc}	57.55^{e}
R.solani-WN293	80.00^{a}	80.00^{a}	64.33^{b}	77.77^{a}	60.66^{c}	58.33^{c}

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).

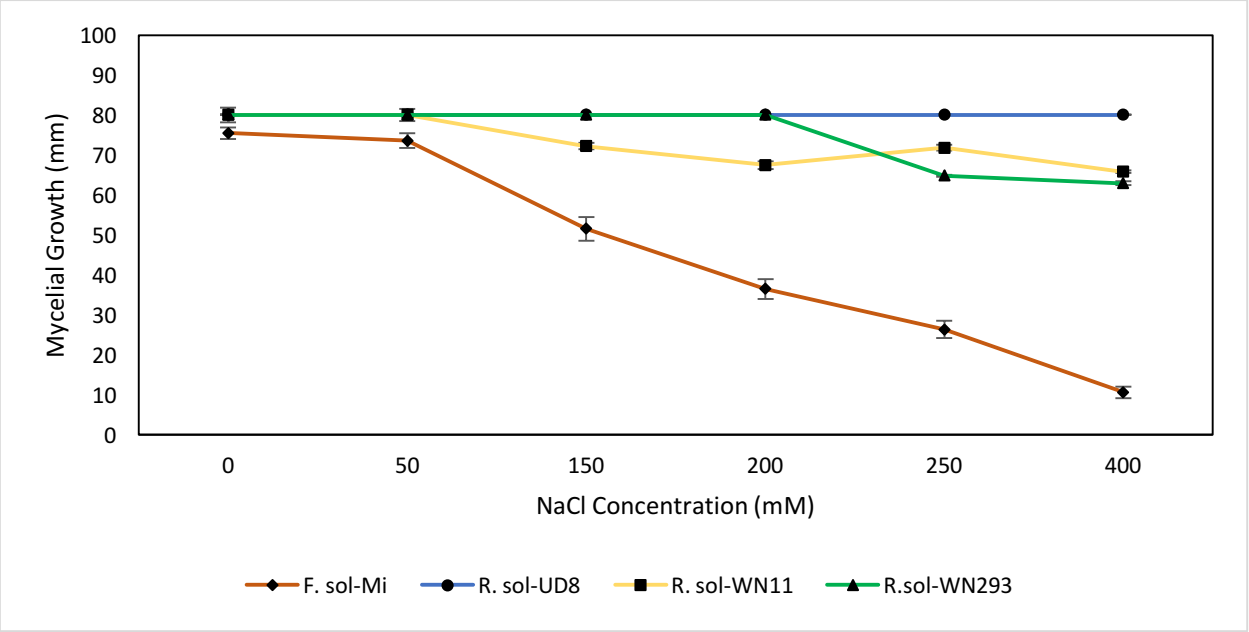

Figure 8: Effect of sodium chloride (NaCl) on the radial mycelial growth measured at day 10 of *F. solani* and *R. solani* isolates that are commonly associated with root rot of common bean. *Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test). Error bars represent standard error of the mean.

Table 7: Salinity effect on mycelial growth at various millimolar concentrations of sodium chloride (NaCl) at day 10

	NaCl Concentrations (mM)					
Pathogen-Isolate	0	50	150	200	250	400
F. solani -Mi	75.44^{a}	73.55^{a}	51.50^{b}	36.44^{c}	26.27^{d}	10.61^{e}
R. solani- UD8	80.00	80.00	80.00	80.00	80.00	80.00
R. solani- WN11	80.00^{a}	80.00^{a}	72.22^{b}	67.44^{b}	71.77^{b}	65.77^{c}
R.solani-WN293	80.00^{a}	80.00^{a}	80.00^{a}	80.00^{a}	64.77^{b}	62.88^{c}

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).

4.2 Fungal Biomass

The isolates of each pathogen had a variation of growth among the concentrations of NaCl.

The effect of NaCl on dry fungal biomass was studied on *F. solani* and *R. solani* using liquid media (Figure 9 and 10). The fungal biomass of the *F.* solani isolate increased at the 50 mM compared to the control and then it had a significant biomass reduction at 150 and 400 mM. The *R. solan*i isolate UD8 demonstrated an increase in biomass from 0 through the 150 mM concentration and then a reduction 400 mM concentration of NaCl. The growth of the other two *R. solani* isolates reduced as the concentration of NaCl increased (Table 8), with significant differences in biomass between the treatments and the control at the 400 mM for WN11 and at all concentrations of NaCl for WN293.

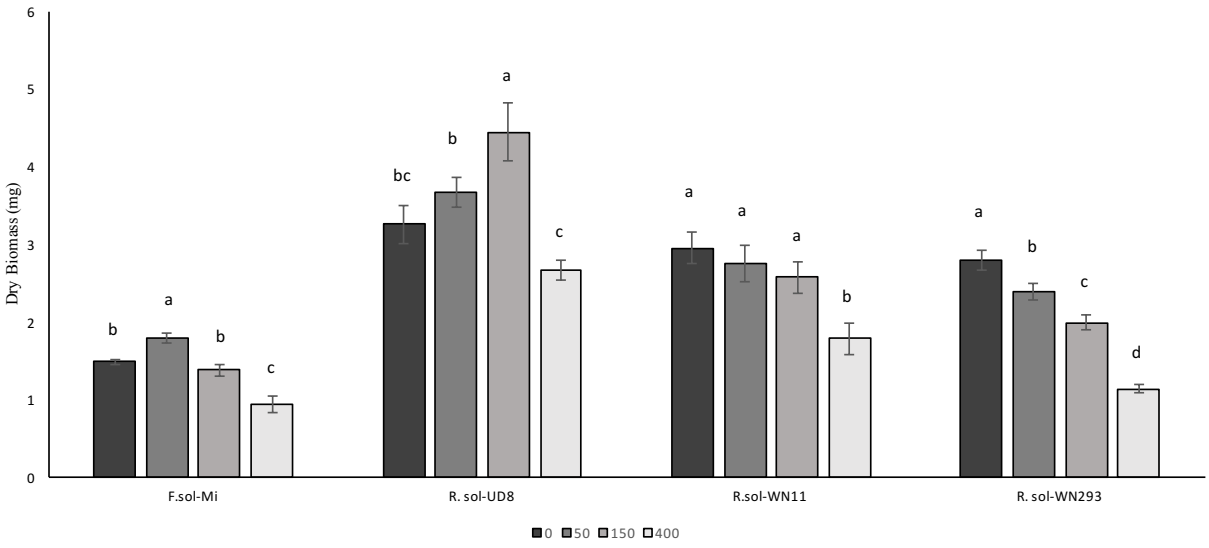

Figure 9: The effect of sodium chloride (NaCl) on the growth (biomass) of *F. solani* and *R. solani* after growing for 14 days in liquid media. *Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test). Error bars represent standard error of the mean.

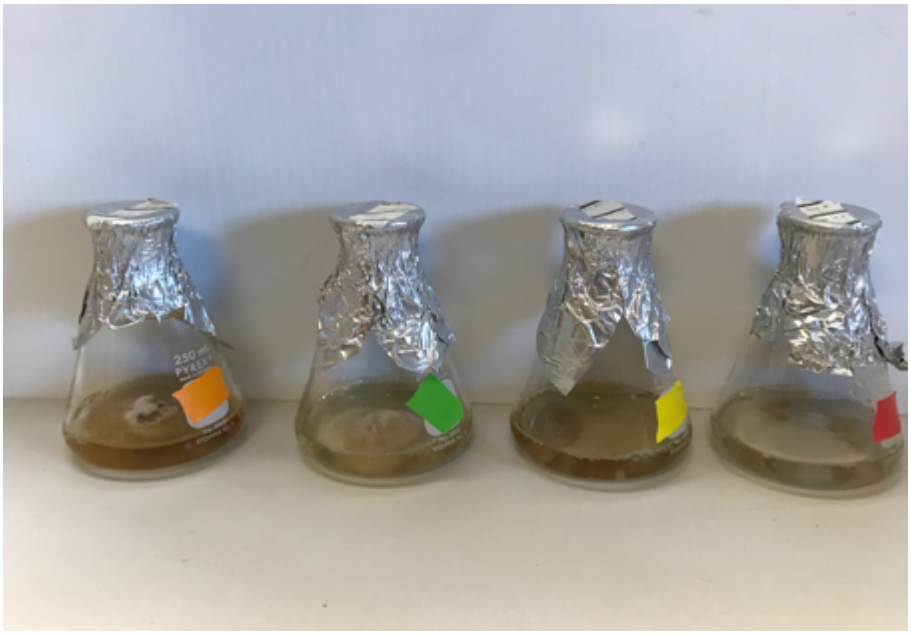

Figure 10: Flasks of amended PDB with *F. solani* cultures after 14 days of shaking. Orange= 0 mM, green= 50 mM, yellow=150 mM, and red= 400 mM of NaCl. From here the biomass is filtered, dried, and weighed.

Table 8: Salinity effects on fungal biomass at various millimolar concentrations of sodium chloride (NaCl) at day 14

Dry Fungal Biomass Weight (mg)*

Pathogen-Isolate	0	50	150	400
F. solani -Mi	148^{b}	178^{a}	137^{b}	92^{c}
R. solani- UD8	325^{bc}	365^{b}	441^{a}	261^{c}
R. solani- WN11	295^{a}	274^{a}	257^{a}	178^{b}
R.solani-WN293	280^{a}	238^{b}	198^{c}	113^{d}

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).

4.3 Spore Germination

Spore germination was assessed by evaluating the number of germinating spores of *F. solani* under increasing NaCl concentrations. The number of germinating spores decreased with

the increasing concentrations although only reduced significantly at 250 mM compared to the control (Table 9 and 10).

Table 9: The effect of salinity effects on spore germination at various millimolar concentrations of sodium chloride (NaCl)

	Spore Germination Counts					
Pathogen-Isolate	0	50	150	200	250	400
F. solani -Mi	37.11[a]	36.23[a]	35.44[a]	33.55[ab]	30.22[b]	0[c]

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).

Table 10: The effects of salinity on the percentage of germinating spores

	Spore Germination (%)					
Pathogen-Isolate	0	50	150	200	250	400
F. solani -Mi	92.77[a]	90.57[a]	88.60[a]	83.87[ab]	75.55[b]	0[c]

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).

4.4 Disease Evaluation

Growth chamber experiments were performed to determine the influence that increasing NaCl concentrations has on root rot and disease severity. The disease severity of *F. solani* and *R. solani* under salt stress significantly increased at each concentration of increasing salt (Table 11). Both pathogens had a significant difference in the amount of rotting, loss of visual root mass, and discoloration at each concentration of salt stress compared to the un-inoculated/un-treated control (Figure 8 and 9). Inoculated roots without salt had a lower disease rating compared to the salt treated and inoculated roots.

Table 11: The effect of disease severity under salt stress

	Transformed Mean Rate (R*)	
NaCl Conc (mM)	*F.solani*	*R.solani*
Np/Ns	0^{d}	0^{d}
0	1.21^{c}	2.79^{c}
50	4.66^{b}	4.66^{b}
100	6.78^{a}	4.66^{b}
150	6.78^{a}	6.78^{a}
200	6.78^{a}	6.78^{a}

*Means followed by the same letter are not significantly different (α=0.05) level (Fisher's LSD test).
R* Disease rating for each replication (R) was transformed to R* = (R1.5 – 1)/1.5 in order to obtain a linear scale and an approximately normally distributed variable with constant variance. Analysis of variance (ANOVA) and least significant difference (LSD) were based on R*

Figure 11: Common bean plants of the cultivar 'Redhawk' inoculated with *F. solani*-Mi-Mic-B8 using the paper towel and sand corn meal method and treated with NaCl at concentrations of 50, 100, 150, and 200 mM. Roots were evaluated 10 days after inoculation (DAI). From left to right: No Pathogen + No salt, pathogen + no salt, pathogen + 50 mM, pathogen + 100 mM, pathogen + 150 mM, and pathogen + 200 mM.

Figure 12: Common bean plants of the cultivar 'Redhawk' inoculated with *R. solani*-WN293 using the paper towel and sand corn meal method and treated with sodium chloride NaCl at concentrations of 50, 100, 150, and 200 mM. Roots were evaluated 10 days after inoculation (DAI). From left to right: No pathogen + No salt, pathogen + no salt, pathogen + 50 mM, pathogen + 100 mM, pathogen + 150 mM, and pathogen + 200 mM.

CHAPTER V: DISCUSSION

Common bean production is vital for human consumption and farmers' incomes in many countries around the world. However, common bean is production is often challenged by many abiotic and biotic stresses including changing environmental conditions and pest pressure. Among diseases affecting common bean production, root rots are of global significance and can cause up to 42-88% yield loss for *R. solani* (Beebe *et al*., 1981; Tan & Tu, 1995) and up to 100% for *F. solani* (Mukankusi *et al*., 2011). Root rot diseases of common bean can be caused by several pathogens present either alone or in complexes and among them *Fusarium solani* and *Rhizoctonia solani* are two of the most important fungal species associated with root rot (Mathew *et al*., 2012; Valentín Torres *et al*., 2016; Nasari & Mousavi, 2015). Soil salinity which can pose as a major abiotic stress in crop production is a growing problem worldwide (Shrivastava & Kumar, 2015) that is affecting dry bean production areas as well (Gama *et al*., 2007).

Currently there are no common bean varieties with complete resistance to root rot, and growers primarily rely on partial resistance, cultural practices and the use of seed treatments to manage the disease. As salinity is a major abiotic stress factor, varietal screening of common bean for enhanced salt tolerance is an ongoing effort (Ndakidemi & Makoi, 2009). In nature, biotic and abiotic stresses often occur together and the effect of one type of stress could be compounded or diminished by the presence of another. However, there have been limited studies conducted on evaluating the effect of a combination of biotic and abiotic stresses with no reports currently available for such studies in common beans. Roots and pathogens are likely to be most vulnerable to the direct effects of increased soil salinity but currently, there are no reports on the influence of salinity on root rot pathogens like *F. solani* and *R. solani* infecting common bean. Studies conducted on asparagus have shown that using sodium chloride as a soil amendment reduced the disease severity of *Fusarium* crown and root rot caused by *Fusarium oxysporum* and *Fusarium*

proliferatum (Elmer, 1992) and suppressed *F. oxysporum* mycelial growth and conidia germination in a study by Amir *et al*., (1996). It has also been suggested that when NaCl is used as a biocontrol method, the concentration should not exceed a certain level because over time the pathogen could adapt to the saline conditions would be of danger for plants growing in saline environments (Dikilitas & Karakas, 2014). Our *in-vitro* experiments on the mycelial growth demonstrated that an increase in salinity can affect the rate and pattern of growth of both *F. solani* and *R. solani* though the affect varied between isolates. All four isolates from both pathogens had significant growth reductions compared to the control at all three time-points except the *R. solani* isolate UD8 which reached maximum possible growth after the initial day 3 evaluation when it demonstrated reduction. In addition to the differences in growth rate, visible differences in the mycelial density of *R. solani* isolates was observed as the rates of NaCl increased. The mycelial growth was seen to be less dense and appeared to demonstrate aerial growth as if the hyphae were trying to grow away from the toxic substrate in plates with higher NaCl concentrations of 150 mM through 400 mM. The isolates of *R. solani* also produced survival structures, sclerotia, during the later days of measurement. The *F. solani* isolate demonstrated a change in mycelial color at the 150 mM concentration and continued to the 400 mM concentration.

To further investigate the changes in mycelial growth and assess the effect of NaCl on biomass, fungal plugs were grown in liquid potato dextrose broth (PDB) amended with NaCl at different concentrations. As the salt concentration in the solution increases, the water potential is subject to change exposing the organism to different osmotic and water potentials as it would in saline soils where they respond by adapting to the low water potential or reduce their biomass unit activity (de Souza Silva, 2012). This was seen in our studies where the growth was decreased by reducing fungal activity or increased by the fungal adaption. An addition of NaCl to the liquid media resulted in changes in biomass in each of the four isolates, but the effect of NaCl appeared

to vary between isolates and there was no consistent trend except that the biomass at the highest concentration of 400 mM NaCl was significantly lower than the control for all except the *R. solani* isolate UD8. The *R. solani* isolate WN293 appeared to be the most affected and had significant reduction in biomass compared to the control at all the four concentrations whereas UD8 was the least affected, and showed an increase in biomass through the concentrations until it was reduced at 400 mM NaCl but the reduction was not significantly different from the control.

According to earlier reports, highly saline conditions are responsible for fungi having reduced mycelia, conidia formation, and sporulation due to the low osmotic potential, toxicity and reduced nutrient availability (Jones *et al*., 2011; Egamberdieva, 2012). Members of the widespread *Fusarium* genera including *F. solani*, have been studied previously to be moderately halotolerant (Mandeel, 2006). Fungi under a low osmotic potential change their morphology, hyphal growth, and there is an overall decrease in spore germination (Juniper & Abbott, 2006). In our studies we observed aerial hyphal growth, and less dense mycelia at the 150 mM and higher concentrations for *R. solani* as well as a decrease in the spore germination percentage for *F. solani*. It has been previously reported by Oren (2001) and Hagemann (2010) that sensitive cells under low osmotic potential have the ability to produce osmolytes (polyols) that help preserve water (Beales, 2004) thus using more ATP energy that is required for growth (Oren, 1999). In our studies, pathogen mycelial growth and biomass was initially increased under higher concentrations of salt compared to the control in three of the four isolates, such increase of *F. solani* mycelial growth by NaCl was also observed by Firdous and Shazbad (2001) where they reported an increase in mycelial growth under 100,000 ppm of NaCl, much higher than what most crop plants can thrive under. Also, Porter and Adamson (1993) observed that *Cercospora* leaf spot of peanuts was favored by soil salinity. A positive effect on fungal growth and conidia germination under 50 mM of NaCl was suggested by Dikilitas (2003) whereas Turco *et al*. (2002) saw an increase in cell wall enzymes and conidia

formation that lead to an increase of virulence of fungal pathogens. The combination of the salt, pathogen, and plant were evaluated in the above study for disease severity.

Our findings suggest that increased salinity can affect fungal growth. This effect is likely to vary not only between pathogens, but also between isolates of each pathogen, and could be positive with an increase in growth up to a certain concentration of NaCl or negative. There is also a possibility of change in morphology of the cultures when the fungus is grown in the presence of excess NaCl. However, the *in-planta* evaluation of the effect of increased salinity on disease development due to the *F. solani* and *R. solani* isolates used in this study demonstrates that in spite of the differences in the effect of salinity on fungal growth between the isolates, root rot severity is likely to be increased with the increase in salinity. These findings agree with previous publications where it is mentioned that if the salinity level is tolerable by the plant, severity may be increased by the additional stress if the pathogen is slightly or not affected by the salt at all (Hassan & Shahzad, 2004; Goudarzi & Pakniyat, 2008; Saadatmand *et al*., 2008). Even if the fungi are affected by the salt stress, it is understood that the tolerance of most crop plants is far less than what fungi can tolerate (Attaby, 2001; Nayak *et al*., 2012). This is similar to our study on the effect of salinity on fungus as compared to the tolerance of bean reported by Läuchli (1984), where common bean suffers yield loss at concentrations of soil salinity as low as 2 dS m^{-1}. Therefore, we conclude that the combined effect of pathogen and salt on the plant may pose to be a more serious threat to the bean plant than the salt or pathogen alone.

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