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Induced Systemic Resistance Against Nematodes by Plant Growth-Promoting Microbes in Potatoes

Cole Grannan
University of Northern Colorado

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

INDUCED SYSTEMIC RESISTANCE AGAINST NEMATODES BY PLANT GROWTH-PROMOTING MICROBES IN POTATOES

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Cole Grannan

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Accepted by the Thesis Committee:

_____
Karen Gomez, Ph.D., Research Advisor

_____
Emily Holt, Ph.D., Committee Member

_____-
Mohamad Chikh-Ali, Ph.D., Honorary Committee Member

Accepted by the Graduate School

_____
Jeri-Anne Lyons, Ph.D.
Dean of the Graduate School
Associate Vice President for Research

ABSTRACT

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Biofertilizers consist of plant growth-promoting microorganisms that establish symbiotic relationships with plant roots and stems. These beneficial microbes play a crucial role in enhancing plant defenses against various threats including insects, nematodes, and microbial pathogens. The purpose of this study was to investigate the effectiveness of two fungal biofertilizers (an endophytic strain of Beauveria bassiana (BA) and a mixture of arbuscular mycorrhizal (AM) fungi), specifically their ability to induce systemic resistance against plant-parasitic nematodes (PPNs) in potatoes (Solanum tuberosum cv. Masquerade) under controlled greenhouse conditions. The study utilized soil collected from Colorado State University's San Luis Valley (SLV) Research Center where potato tubers exhibited severe symptoms of corky ringspot disease caused by tobacco rattle virus (TRV), which is transmitted by PPNs. Potato tubers were planted in both control soil and SLV soil with and without biofertilizer inoculation. After 12 weeks (approximately three months) of growth, the researchers assessed plant growth parameters and nematode presence. Surprisingly, the SLV soil negatively impacted all measured plant growth parameters. Additionally, the endophytic strain of *B. bassiana* adversely affected tuber numbers in control soils. These findings highlighted the need for further investigation into the potential use of *B. bassiana* and AM fungi as biocontrol agents.

Keywords: biofertilizers, nematodes, plant growth-promoting microbes, and potatoes

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Statement of Purpose

The purpose of this study was to investigate the effectiveness of two fungal biofertilizers: an endophytic strain of Beauveria bassiana (BA) and a mixture of arbuscular mycorrhizal (AM) fungi, specifically their ability to induce systemic resistance against plant-parasitic nematodes (PPNs) in potatoes (Solanum tuberosum cv. Masquerade) under controlled greenhouse conditions. The research evaluated the impact of these biofertilizers on plant growth and the presence of nematodes in soil samples from the San Luis Valley (SLV), Colorado, an area where corky ringspot disease, caused by the tobacco rattle virus (TRV), significantly affects potato crops. The study aimed to determine whether these biofertilizers enhanced resistance to PPNs, contributing to sustainable agricultural practices and improved crop yields.

Aims

Plant growth-promoting microorganisms (PGPMs) are used in organic agriculture to enhance crop yield and health. These beneficial microorganisms are known to increase the nutrient and water uptake of the plants and/or boost the plant's resistance to pests and pathogens. Certain fungi are classified as PGPMs because they can form an endophytic symbiosis with the host plant and manipulate its pathogen and herbivore-induced defenses via the salicylic acid and/or jasmonic acid pathways. However, the benefit of using such fungi to control soil pests and pathogens in potatoes remains elusive. This research investigated whether two commercially available fungal biofertilizers improve potato resistance against plant parasitic nematodes. The

first biofertilizer is composed of a portfolio of arbuscular mycorrhizal (AM) fungi (*Glomus intraradices*, *Glomus mosseae*, *Glomus aggregatum*, and *Glomus etunicatum*) that have been extensively investigated in other crop systems for their role in regulating plant host defenses against pathogens and herbivores (Bernaola & Stout, 2019; Diagne et al., 2020; Pieterse et al., 2014; Rivero et al., 2021). The second biofertilizer contains the fungi *Beauveria bassiana*, which has been minimally investigated for its manipulation of plant defenses but is frequently used to control pests in organic agriculture systems. Soil samples were collected from the Colorado State University San Luis Valley Research Center (SLV) fields, where symptoms of plant parasitic nematode activity were observed in tubers of the previous year's crop of the potato cv. Masquerade. Biofertilizers were applied to pots planted with tubers (*Solanum tuberosum*) of the same cultivar, i.e., cv. Masquerade and allowed to grow under greenhouse growing conditions to evaluate the following objectives and test the associated hypotheses:

- Objective 1: To evaluate the impact of biofertilizer application on plant growth.

  H1 Applying SPE-120 will improve shoot mass, height, tuber number, yield, and diameter over the water treatment.

  H2 Applying MycoApply Endothrive will improve shoot mass, height, tuber number, yield, and diameter over the water treatment.

- Objective 2: To study the impact of biofertilizer application on soil nematode presence.

  H3 The application of SPE-120 will negatively impact the density of soil nematodes over the water treatment.

  H4 The application of MycoApply Endothrive will negatively impact the density of soil nematodes over the water treatment.

Overview of Biofertilizer Use for the Control of Pests in Potatoes

Potatoes (*Solanum tuberosum*) are an economically important vegetable crop for Colorado agriculture. In 2021, Colorado produced over 2.1 billion pounds of potatoes valued at $217 million, making potatoes the sixth-largest vegetable crop in the state (U.S. Department of Agriculture et al., 2022). Potatoes account for 10% of the total crop income and 48% of organic crop sales revenue, with potato cultivation taking place on 54 thousand acres of land in Colorado (U.S. Department of Agriculture & National Agricultural Statistics Service, 2020). The potato crop is greatly affected by animal pests such as insects, mites, nematodes, rodents, slugs, snails, and birds. Estimates in 2005 indicated pests caused an 11% annual loss of the worldwide potato crop (Culliney, 2014; Oerke, 2006). When pests such as the stubby-root nematode (SRN) infect these crops, they can significantly impact potato revenue. Stubby-root nematodes are plant-parasitic nematodes that feed on plant roots, stunting root growth and transmitting tobacco rattle virus (TRV), which causes corky ringspot disease (CRS). Stubby root nematode infestations can severely damage tuber quality, causing the tubers to be rejected from the market if damage reaches 5-10% (Hafez et al., 2020). In Colorado, SRNs are a significant concern for potato growers due to their potential impact on the financial return from infected potato fields. Estimates of economic losses, specifically from SRNs, are lacking (Mumford et al., 2000; Yan et al., 2016). Modern control of SRNs uses the same management methods for all plant parasitic nematodes (PPNs). These methods include the sanitation of equipment and soil, planting potato cultivars resistant to corky ringspot disease, and the application of nematicides to soil (Trivedi & Barker, 1986).

As organic farming continues to expand in the United States and worldwide, fungal biofertilizers containing plant growth-promoting microbes (PGPMs) are gaining popularity

(Bhattacharjee & Dey, 2014; Whipps, 2004; Willer & Lernoud, 2018). Commercial products contain PGPMs that establish symbiotic relationships with the crops and application to growers' seeds and farming soil has been shown to increase plant nutrient uptake, enhance yield, and promote plant growth (Wu et al., 2005). Recent research indicated that some root-associated fungi and bacteria could stimulate host plant immune responses against nematodes by inducing systemic resistance throughout the plant (Castillo et al., 2006; De La Peña et al., 2006; Mwaura et al., 2017). The induction of systemic resistance in plants occurs when a microbial symbiont modulates the defense signaling pathways in its host plant, augmenting the plant's response to biotic and abiotic stresses.

Plant Parasitic Nematodes in the United States

Plant parasitic nematodes (PPNs) are microscopic, unsegmented worms in various environments worldwide. They infect and damage crops, such as potatoes, causing approximately 4% of yield loss of potatoes annually in the United States (Bongers & Bongers, 1998; Koenning et al., 1999). These nematodes utilize a stylet, a small sharp tube, to puncture the cells of root surfaces and ingest the cellular contents, thereby hindering the growth and fitness of the host plant (Oka et al., 2000). Major PPNs including the root-knot (*Meloidogyne*), cyst (*Globodera*), stubby-root (*Nanidorus*, *Paratrichodorus*, and *Trichodorus*), dagger (*Xiphinema*), and root-rot (*Ditylenchus*) nematodes are known to infect potatoes in the United States (Abrantes et al., 2023). Nematode damage decreases potato yields directly by ingesting their hosts' cellular contents and indirectly by transmitting viruses that lower the quality of tubers (Yan et al., 2016). Plant parasitic nematodes can transmit *Nepoviruses* and *Tobraviruses*, which infect plant tissues and cause plant tissue chlorosis and necrosis (Singh et al., 2020).

Common Plant Parasitic Nematodes in the United States

Root-knot nematodes (*Meloidogyne* spp.), devastating pests of potatoes, were first detected in the United States in 1889 in Florida (Neal, 1889). *Meloidogyne incognita*, one of the most well-known root-knot nematodes, is a generalist parasite that infects crops worldwide (Abad et al., 2008). These nematodes are identified by the galls or knots of enlarged cells that form on the plant tissue due to their feeding. These growths occur when the nematode injects esophageal secretions, which regulate the plant cell's ability to divide and expand (Perry et al., 2009). Root-knot nematodes can reproduce sexually or asexually. During sexual reproduction, females inject eggs into the galls formed during feeding, further damaging the plant. This damage causes stunting of the plant's stem tissue, suppressing growth and reducing yield (Hafez et al., 2020; Wesemael et al., 2014). Management strategies include crop rotation, soil fumigation, and resistant plant varieties. While chemical nematicides such as methyl bromide and 1,3-dichloropropene are effective, they pose environmental and human health risks (Desaeger et al., 2017). Organic management strategies include the use of biofumigants, solarization, and organic amendments (Desaeger et al., 2017).

Cyst nematodes (*Globodera* spp.) represent another worldwide pest that adversely impacts potato production. An example of a cyst nematode is the *Globodera rostochiensis*, first identified in New York, United States, in 1940 (Dandurand et al., 2019). These nematodes feed by injecting esophageal solutions into the root tissue of the plant, which results in a single large, multinucleated cell known as a cyst (Chandran & Wildermuth, 2016). The formation of the cyst inhibits the growth of the host plant and can induce senescence, potentially reducing potato yield by up to 80% (Dandurand et al., 2019). Female *Globodera* nematodes develop within a cyst, feeding and growing until they mate with a male to reproduce sexually. They then develop eggs

that exit the cyst when hatched (Clarke & Hennessy, 1984). Cyst nematodes feed on plants in the Solanaceae family and are found throughout the United States (Skantar et al., 2007). Strategies such as crop rotation, resistant plant varieties, and soil fumigation with nematicides effectively manage cyst nematodes. Organic options, such as solarization and organic amendments, are also used (Evans, 1993; Krueger & McSorley, 2008).

Stubby-root nematodes (SRNs) parasitize the root system of over 400 crop species, including potatoes (Subbotin et al., 2019). The first SRN identified in the United States was *Trichodorus allius* in Oregon in 1963 (Jensen, 1963). The stubby-root nematodes belong to the *Trichodorid* and *Paratrichodorid* genera (Riga et al., 2007). These nematodes are ectoparasites, feeding on the root surface and reproducing externally from the plant (Hunt, 1993). Stubby-root nematodes reproduce sexually and parthenogenically. The females will migrate through the soil using root exudates they encounter to navigate to ideal hosts (Mathew & Opperman, 2020). The parasitism by this nematode on the plant severely hinders the growth of the host because its feeding causes the roots to be stunted, leading to decreased nutrient and water uptake (Yan et al., 2016).

Viruses Transmitted by Plant Parasitic Nematodes

Plant parasitic nematodes can be vectors of two genera of viruses: *Nepovirus* and *Tobravirus* (Singh et al., 2020). Nepoviruses are positive-stranded RNA viruses composed of two separate RNA genomes packed into polyhedral particles transmitted by PPN (Sanfaçon, 2021). One *Nepovirus* found in Colorado that infects potatoes is the tomato ringspot virus (Nachappa et al., 2020). Plant parasitic nematode species in the genus *Xiphinema* (dagger nematodes) transfer this virus throughout the root and stem tissues of the host plant, causing chlorosis and necrosis (Nachappa et al., 2020; Rush & Gooding, 1970). The stubby-root nematodes are vectors for TRV,

which is a species in the *Tobravirus* genus in the family *Virgaviridae* with a bipartite positive sense single-stranded RNA genome encapsulated in tubular particles (MacFarlane, 2008).

The genome of TRV consists of a longer RNA, RNA1 genome, and a smaller RNA, RNA2 genome within their viral particles (MacFarlane, 2008). The RNA1 genome contains the nucleotide sequences for virus replication, movement, and RNA silencing suppression, while the RNA2 genome encodes for the coat protein and nematode transmission proteins (MacFarlane, 2008). A standard method to quantify the transmission success of these viruses is via reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), which involves the isolation and amplification of a conserved sequence of RNA1 from infected plant tissue (Mumford et al., 2000). The TRV infection causes corky ringspot disease (CRS) in potato tubers, expressed as external and/or internal necrotic concentric rings and arcs (van Griethuysen, 2020; Yan et al., 2016). The transmission of viruses vectored by nematodes decreases the quality of potato tubers and renders them unmarketable. Therefore, it is essential to identify the presence of SRN and other PPNs in the soil to mitigate the impact of CRS on crop quality and financial returns (Yan et al., 2016).

Current Methods of Control of Plant Parasitic Nematodes

To effectively control nematodes, it is important first to evaluate their presence in the soil. This can be done by identifying and quantifying them using ribosomal DNA 28S, 18S, 5.8S, internal spacer regions, or by staining the nematode-infected tissues in and around the plant roots. Once the presence of nematodes is confirmed, specific control methods can be tailored and implemented using physical soil manipulation or chemical solutions (Sasanelli et al., 2021). Nematode populations can be managed by reducing their reproduction (Bybd et al., 1983; Huang et al., 2018; Subbotin et al., 2019).

Management methods are administered after the presence of PPN in a field or soil is confirmed. These methods can be broadly classified into cultural, biological, and chemical techniques. Cultural methods involve the application of practices such as soil sanitization, selecting a plant-parasitic nematode-resistant crop rotation regime, and using organic amendments like flooding and fallowing (Krueger & McSorley, 2008; Winchester, 1964). Biological methods use microorganisms such as plant growth-promoting organisms or antagonists to control the fitness of PPN (Pires et al., 2022). Chemical methods, on the other hand, involve the use of nematicides or other compounds to inhibit the health and reproduction of PPN (Pires et al., 2022). Integrated pest management programs involve planning, monitoring, prevention, and control to effectively manage PPN by incorporating threshold-based response (Abbas et al., 2022).

Proper equipment and substrate sanitation are the first defenses against introducing PPN. Removing dirt and dried sediment from the equipment and using uncontaminated soil minimizes the introduction of PPN (Mitiku, 2018). Soil is sanitized using methods of anaerobic soil disinfestation. This entails flooding, solarization, or fallowing. The efficacy of these methods in reducing stubby-root nematodes is variable (Molendijk & Hoek, 2005; Molendijk et al., 2009). For example, the flooding sanitization method is a popular cultural method that involves releasing water over a field to create anaerobic soil conditions (Trivedi & Barker, 1986). However, the migratory behavior of stubby-root nematodes renders this method less effective as they move to nearby alternative plant hosts (Asjes et al., 1996). One widely used cultural control method semi-effective against SRN is fallowing, where a field is plowed, and active plant matter is removed mechanically or chemically (Sasanelli et al., 2021; Weingartner et al., 1993). Solarization is a method of increasing soil temperature beyond the survivable range of most

microbes by covering a field with a layer of plastic. This control method has also been semi-effective in regulating SRN species (Chellemi et al., 1993).

Crop rotation is a popular cultural method for managing pests. However, its effectiveness on stubby-root nematodes is limited compared to other PPNs (Anderson et al., 2016). This method involves selecting pest-resistant cultivars and rotating their planting across growing seasons (Trivedi & Barker, 1986). The selected crops are resistant to the pest, allowing them to tolerate the effects and decrease the presence of the pest in the soil without suffering severe damage or yield loss (Teetes, 1996; Winchester, 1964). An example of resistance in crops is *Asparagus officinalis*, which produces a toxic glycoside harmful to Trichodorid nematodes (Rohde & Jenkins, 1958). Resistant potato cultivars show minimal CRS damage to their tubers by the SRN vectored TRV. A few resistant potato cultivars include Bintje, Centennial Russet, and Ciklamen (Yellareddygari et al., 2018). There are limitations to using crop rotation to manage SRN presence because stubby-root nematodes migrate between host plants and have a wide range of potential hosts, utilizing weeds or other plants growing in the field as alternative hosts. The SRNs can also reproduce in the soil and not exclusively in the host plant's tissue. These two factors make crop rotation a limited method of control because of the life cycle of the SRN (Anderson et al., 2016). Control of SRNs is not limited to human influence via crop rotation and sanitation. Another method involves manipulating the plant immune response by adding biofertilizers and inducting systemic resistance.

Plant Immune Response to Parasitic Nematodes and Counter Responses

Understanding how a plant defends itself from pests and pathogens is essential knowledge for conservation and agriculture. Various defense mechanisms have evolved in plant immune systems responding to biotic and abiotic stressors. Plant receptors (pattern-recognition

receptors) interact and recognize molecules associated with pathogens (pathogen-associated molecular patterns), beneficial microbes (microbe-associated molecular patterns, MAMPs), herbivores (herbivore-associated molecular patterns), and mechanical damage (endogenously generated damage-associated molecular patterns) (Pieterse et al., 2014). These modes of plant resistance to abiotic stressors, pests, and pathogens are categorized into three pathways of defense: herbivore-induced resistance (HIR), systemic acquired resistance (SAR), and induced systemic resistance (ISR; Pieterse et al., 2014).

Herbivore-induced resistance (HIR) is a portfolio of defense mechanisms that are activated once a plant recognizes a damage-associated molecular patterns or herbivore-associated molecular pattern. In this pathway, an attacked plant synthesizes the signaling hormones jasmonic acid (JA) and ethylene (ET) throughout its tissues, which then activate defense genes (Pieterse et al., 2014; Turner et al., 2002; Van Loon, 2000). The defense genes play a role in inducing physical and chemical barriers to resist the stressor. Potatoes use physical barriers to defend against PPNs. These include thickening cell walls at invasion sites, making it challenging for nematodes to penetrate cells, and replacing damaged epidermis tissue with cork cambium, thus limiting nematode access to healthy tissue (Kumar & Ginzberg, 2022; Moore et al., 2003; Sun et al., 2020).

Some PPNs can counteract the plant's physical barriers by releasing effector proteins during feeding. Cyst nematodes can downregulate the cell wall-thickening genes (cell wall-associated kinases), increasing feeding efficacy (Chen et al., 2022). Attacked plants produce secondary metabolites that are compounds with diverse toxicological effects against herbivores and local predators (Pirbalouti et al., 2014). Among these secondary metabolites produced for

plant defense are volatile organic compounds, which, when released, can attract or repel herbivores and their predators (Babikova et al., 2013, 2014).

Plants have a primary defense mechanism against pathogens called Pattern/PAMP-triggered immunity (PTI; Ngou et al., 2022). When a plant's PRR recognizes a PAMP, it stimulates local cell death around the infected area. The generation of necrotic tissue around the infected tissue is called the hypersensitive response (HR) and hinders the pathogen's spread to nearby tissues (Dodds & Rathjen, 2010). The PTI triggers the production of the hormone salicylic acid throughout the plant's systemic tissues, which stimulates the transcription of pathogen response genes. These genes produce antimicrobial proteins that increase the resistance of uninfected tissues to the pathogen (Pieterse et al., 2014).

A few potato cultivars, including the Russet Burbank, resist TRV, responding to infection by stimulating necrosis around the infection sites in the tubers and increasing resistance in the whole plant (Palukaitis, 2012). However, plant pathogens can suppress their host’s PTI response, developing virulence factors to thwart the plant’s recognition or defense response (Ghazala, 2007; Pieterse et al., 2014). Plants have developed additional defense mechanisms that detect pathogen effector molecules and trigger a more robust immune response known as effector-triggered immunity to defend against these harmful factors (Dodds & Rathjen, 2010). Effector-triggered immunity and PTI stimulate the buildup of SA throughout the plant in systemic tissues, defined as SAR (Pieterse et al., 2014).

An additional response to pathogens is through the formation of relationships with beneficial microbes. Plants have a long history of forming relationships with microbes, and while some of these interactions have been marked by conflict, others have been founded on mutualism. The evolution of terrestrial plants was made possible by establishing mutualistic

relationships with fungi. Through these relationships, plants could exchange lipids and sugars with PGPMs for greater access to vital nutrients and water, which helped ensure their survival (Bouwmeester, 2021). These complex relationships evolved, leading to nuanced communication and control between the microbes and plant hosts. Induced systemic resistance is a defense mechanism by which a plant's HIR and SAR pathways are modulated by plant growth-promoting fungi and bacteria intertwined with the plant host's tissues. This union primes the plant for a faster and stronger defense response to a pathogen or herbivore by increasing the production of SA and JA (Pieterse et al., 2014). The discovery of ISR led to the use of PGPMs in modern agriculture as biofertilizers and biocontrol agents due to the resulting improved growth in plants that some microbes have on some crops.

Biofertilizers as a Biological Control Method of Plant Parasitic Nematodes

Commercial biofertilizers might contain one or multiple PGPMs but their benefits to the plants are not guaranteed as symbiosis relies on the specific microbes present in the biofertilizer inoculum as well as the attack by pathogens or herbivores (Bhattacharjee & Dey, 2014; Hafeez et al., 2006; Harni et al., 2023; Jorrin & Imperial, 2015; Sanders, 2003; Wu et al., 2005). These microbes are fungi and bacteria that are frequently endophytes, living within a plant's tissues for a portion of their life cycle. When multiple endophytes are associated with a single plant host, the plant can favor the microbe that benefits it most, trading more resources with that symbiont than the others (Jorrin & Imperial, 2015; Sanders, 2003). Fungi are a frequent component of biofertilizers. Arbuscular mycorrhizal (AM) fungi are obligate plant symbionts that form hyphal structures in the root cortex of plants. Entomopathogenic fungi are another plant growth-promoting fungi used to control plant pests. Rhizobia are plant growth-promoting bacteria frequently included as a component of biofertilizers. Commercial biofertilizers can be composed

of a single plant growth-promoting microbe or a combination of fungi and bacteria. The application of biofertilizers as a control method for PPNs introduces a novel means of management practice. Their use and study have been increasing in organic agriculture worldwide (Bhattacharjee & Dey, 2014; Whipps, 2004; Willer & Lernoud, 2018).

Arbuscular Mycorrhizal Fungi as Biofertilizers

Arbuscular mycorrhizal fungi are polynucleate organisms, forming aseptate hyphae throughout the plant root (McGonigle et al., 1990). These hyphae increase the surface area of the plant roots, improving water and nutrient uptake as well as stress response in return for carbohydrates and lipids (Diagne et al., 2020; Lutz et al., 2023). Arbuscular mycorrhizal fungi create branched structures called arbuscules in plant root cortex cells. The arbuscules penetrate cell walls, and the periarbuscular membrane of the cell surrounds the hyphae (During, as cited in Pumplin, 2010). Several species of AM fungi have been observed to activate the SA and JA pathways in model plants, enhancing the yield of certain crops (Babikova et al., 2014; Carrara et al., 2023; Koricheva et al., 2009; Schoenherr et al., 2019; Song et al., 2015).

One of the most well-studied AM fungi, *Rhizophagus irregularis*, has been observed to modulate its host defenses against pathogens and herbivores (Castillo et al., 2006; Schoenherr et al., 2019). In one study by Castillo et al. in 2006, researchers investigated methods of improving the fitness of olive plants (*Olea europaea*) grown in the presence of plant parasitic root-knot nematodes *Meloidogyne incognita* and *Meloidogyne javanica*. They found that those plants colonized by AM fungi (*R. irregularis*) had improved fitness and defense against the root-knot nematodes (Castillo et al., 2006). Schoenherr et al. (2019) observed a similar pattern of defensive priming in potatoes grown in the presence of AM fungi and leaf-chewing caterpillars. The research team was studying how AM fungi regulate defense response genes in potatoes and how

it impacts the fitness of cabbage loopers caterpillars (*Trichoplusia ni Hübner*). The findings revealed that AM fungi can alter the potato's defenses against caterpillars by improving the potato's fitness and reducing the herbivore's fitness (Schoenherr et al., 2019).

MycoApply® is a commercial biofertilizer company that produces products containing portfolios of AM fungi, which are widely utilized to enhance the nutrient uptake of crops. However, few studies have been conducted to evaluate the effectiveness of MycoApply products as biocontrol agents. One study found that applying a MycoApply Endo product improved the resistance of rice (*Oryza sativa*) against borer insects (*Diatraea saccharalis*, *Eoreuma loftini*, *Chilo plejadellus;* Bernaola & Stout, 2019). However, another study found that its application increased the occurrence and damage by verticillium wilt (*Verticillium dahliae*) of peppermint (*Mentha piperita;* Wu et al., 2011). Therefore, biofertilizers containing AM fungi may have varying effects depending on the plant species and the type of fungi.

Entomopathogenic Fungi as Biofertilizers

Entomopathogenic fungi are free-living fungi that can predate or parasitize arthropods. Some entomopathogenic fungi can form relationships with plants, growing inside their leaf and root tissues and promoting plant growth by increasing nutrient uptake (Ownley et al., 2008). *Beauveria bassiana* is a species of fungi in the family *Cordycipitaceae*, which primarily infects insects and other arthropods. However, it is set apart from other entomopathogens because *B. bassiana* forms an endophytic relationship with plants and induces systemic resistance against pathogens and herbivores (Ownley et al., 2008; Wei et al., 2020). *B. bassiana* has two life cycle states. It can freely live in the soil in a saprophytic state, receiving its nutrients from what is available around its mycelium, or, if its septate hyphae encounter a potential host, it will invade the host’s tissue, becoming a symbiont (Youssef et al., 2020). If the potential host it meets is an

insect, the conidia of *B. bassiana* can germinate, developing a specialized structure called an appressorium (germ tube) that enzymatically breaks down the insect's cuticle, allowing the hyphae to penetrate the host (Zhang et al., 2023). Once inside the host, the hyphae grow throughout the organs and tissues, secreting enzymes and toxins to degrade the host's tissues for resources and increase pathogenicity (Zhang et al., 2023).

The invasion of *B. bassiana* will eventually kill the host, after which the fungi return to their saprophytic stage, breaking down the insect's body and producing reproductive conidiophores (Posada-Florez & Vega, 2005). *B. bassiana* can also infect plant tissues, resulting in a relationship similar to mycorrhizal fungi (Ownley et al., 2008; Wei et al., 2020; Zhang et al., 2023). Its mechanism of plant tissue penetration is the same as what it utilizes for insects, forming a germ tube that allows its hyphae to grow inside the root and stem tissues of the plant. *B. bassiana* increases the nutrient availability for the plant, forming this relationship in exchange for plant carbohydrates (Moonjely et al., 2018). The application of *B. bassiana* thus promotes plant growth by ISR and feeding on the arthropods that interact with the plant. This has recently led to their extensive use as a component of biofertilizers and biocontrol agents.

Entomopathogenic fungi are effective at combating the potato parasitic potato tuber moth (*Phthorimaea operculella*), Colorado potato beetle (*Leptinotarsa decemlineata*), and root-knot nematodes (*Meloidogyne incognita;* Baki et al., 2021; Youssef et al., 2020; Zhang et al., 2023). The degree of how beneficial *B. bassiana* is as a biocontrol method is confounded by their attraction of nearby plant parasitic nematodes to the plant host, which increases the density of potato parasitic nematodes in the rhizosphere, increasing the frequency of parasitism of the tubers and root tissues by the nematodes (Mwaura et al., 2017). One commercial biocontrol agent that contains *B. bassiana* that is being used in a potato farm in Northern Colorado is SPE-

120 (H. Strohauer, personal communication). Little research has been done on the SPE-120 strain of *B. bassiana* specifically, but it has been observed to improve radish (*Raphanus sativus*) emergence in fields blighted by cabbage maggots (*Delia radicum;* Buckland & Rasmussen, 2022).

Rhizobia Bacterium as Biofertilizers

Bacteria in the genus *Rhizobium* are root endophytes, creating nodule structures inside the root. The growth of these nodules allows the bacteria inside them to fix nitrogen from the environment. The plant trades carbohydrates for the nitrogen captured by the rhizobia (Trinick, 1980). Rhizobia has been observed to induce systemic resistance in various plant hosts against pathogens and herbivores (Castillo et al., 2017; Hasky-Guenther et al., 1998; Pangesti et al., 2016; Reitz et al., 2000). Pangesti et al. (2016) performed a study investigating ISR in thale cress (*Arabidopsis thaliana*) by the rhizobia *Pseudomonas simiae* WCS417r against cabbage moth (*Mamestra brassicae*). The researchers examined how ISR regulated the expression of two JA defense genes (MYC2 and ORA59). They found that the bacterial ISR negatively affected fitness by decreasing the larval weight of the herbivorous moths by modulating the activity of the two defense genes by triggering the JA and ethylene defense pathways (Pangesti et al., 2016). Rhizobia has also been observed to trigger systemic resistance against pathogen-vectoring organisms like the potato cyst nematode *Globodera pallida* (Hasky-Guenther et al., 1998; Reitz et al., 2000).

Significance

In Colorado, most potatoes are produced in the San Luis Valley (SLV), an agricultural region in south-central Colorado (Ehrlich et al., 2020). The income of potato growers there is directly affected by local crop pests and pathogens. Because of this fact, it is integral for potato

research to that mirror the growth setting of potatoes in SLV. Therefore, this study aimed to investigate whether biofertilizers could augment the resistance of potatoes to plant parasitic nematodes. The soil substrate for this study was collected from a grower's field in the SLV with a TRV infection history. The Masquerade potato cultivar stock, sensitive to CRS, was also collected from the Research Center and used in the study. The biofertilizers used in this study contained AM or entomopathogenic fungi (Terregena®SPE-120, MycoApply®Endothrive) that form endophytic relationships with potato roots and are common components of biofertilizers (Bhattacharjee & Dey, 2014; Hartley & Gange, 2009; Ownley et al., 2008). The efficacy of the biofertilizers was quantified using measurements of plant growth, soil nematode presence, and hyphal colonization of the plant roots.

CHAPTER II

METHODOLOGY

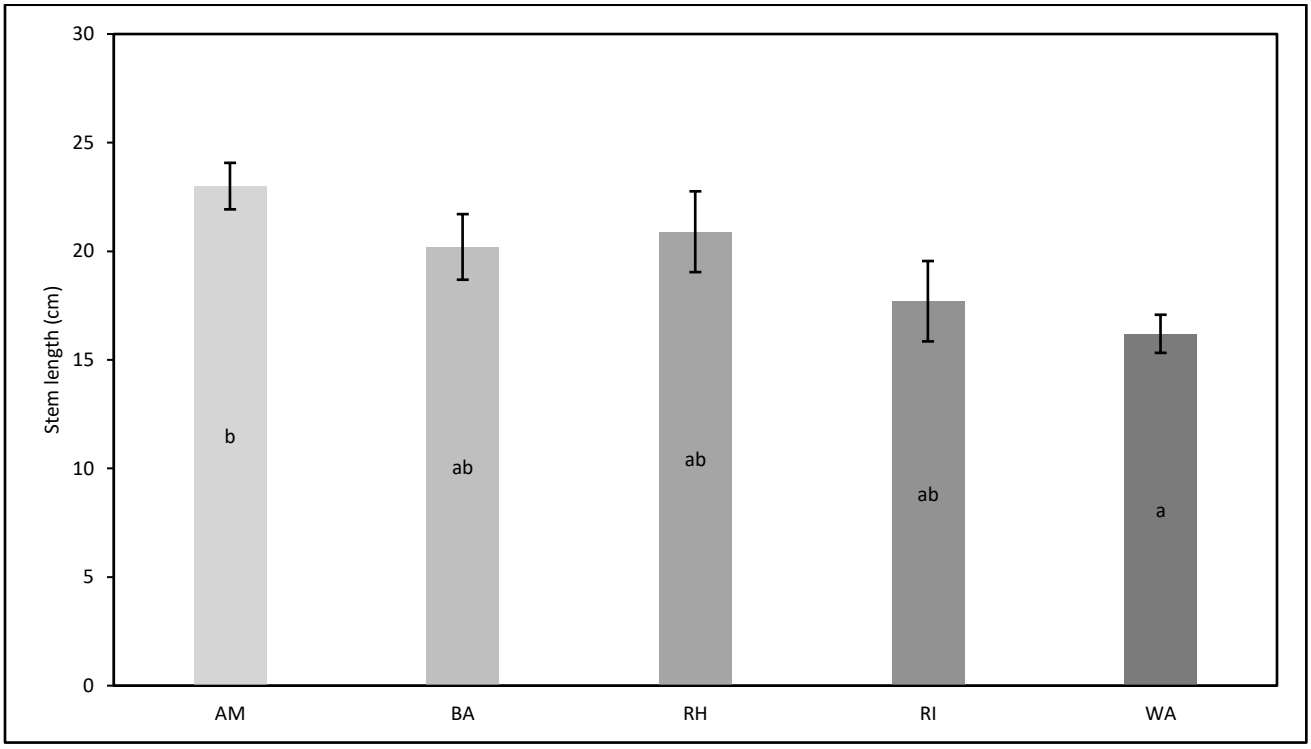
Summer 2022 Pilot Field Study

During the summer of 2022, a pilot study was conducted in a commercial potato field in La Salle, Colorado. The aim of this pilot was to investigate the effects of various biofertilizers on the fitness of potatoes grown in soil where corky ringspot disease symptoms were observed and to determine which biofertilizers would be used for the 2023 greenhouse study. The biofertilizers tested included Terregena®SPE-120 (BA), MycoApply®Endothrive (AM), Advanced Nutrients® Voodoo Juice PLUS tablets (RH), AGTIV® Potato Liquid (RI), and a water control (WA). MycoApply®Endothrive is composed of a liquid solution that contains the AM fungi species *Glomus intraradices, Glomus mosseae, Glomus aggregatum,* and *Glomus etunicatum,* each at 381 propagules/ml. MycoApply® Endothrive was diluted with water according to the company's recommendation in furrow application with a concentration of 0.629 ml/l. Terregena®SPE-120 (BA) is composed of *Beauveria bassiana* at 5.6 x 10^3 propagules/ml. BA was diluted with water to the commercially recommended concentration of 6.25 ml/l. The soil was drenched with 300 ml of BA/water solution. Nutrients® Voodoo Juice PLUS (RH) tablets are composed of a portfolio of plant symbionts and were dissolved and applied at a rate of 0.1 g/gal. AGTIV® Potato Liquid (RI) is composed of a liquid solution with *Rhizophagus irregularis* at 250 propagules/ml. AGTIV® Potato was diluted to its in-furrow application rate of 4.98 ml/l. Tap water (WA) was used as a control and was applied at a 5 ml volume. All treatments were applied at the recommended concentrations to Banana Fingerling potato tubers (used as seed)

and soil during furrow planting (May 14, 2022). Stem length from the base of the stem to the apical meristem was measured weekly for nine weeks up to harvest to measure the impact of these treatments on potato growth. An analysis of variance (ANOVA) was used to analyze the individual effects of biofertilizers on the final stem length. The ANOVA revealed a significant difference in growth between the treatment groups ($df = 4$, $p = 0.0165$). A Tukey post hoc analysis showed that the stem length of plants treated with MycoApply®Endothrive was significantly greater than those treated with water ($p = 0.0146$); whereas all other treatments did not differ in growth outcomes compared to water controls nor MycoApply Endothrive (see Figure 2.1). The results suggested that AM fungal biofertilizers could enhance potato stem length in soil containing plant parasitic nematodes. However, it is important to note that unforeseen events during the field season impacted plant growth, subsequently influencing the final yield data collected on August 12, 2022. Despite these challenges, the study provided valuable insights into the potential benefits of using biofertilizers to improve crop health in challenging soil conditions and specifically aided our selection of a subset of biofertilizers for the greenhouse study.

Figure 2.1

Summer 2022 Field Experiment



Note. Stem length of plants treated with MycoApply Endothrive (AM), SPE-120 (BA), Advanced Nutrient Voodoo Juice (RH), AGTIV Potato L (RI), and water (WA). Treatments that share the same letter are not significantly different according to Tukey's post hoc test ($p < 0.05$). Values *represent the* mean ± SE, $N = 75$.

Greenhouse Experimental Setup 2023

The greenhouse experiment began on May 16, 2023, and potatoes were harvested on August 25, 2023. Potato (*Solanum tuberosum* L.) cv. Masquerade tubers were obtained from the Colorado State University (CSU) San Luis Valley Research Center, Center, CO, United States. The plants were grown in a climate-controlled greenhouse at the University of Northern Colorado, Greeley, CO, United States. The temperature and humidity in the greenhouse were set to 22-24 °C and 65% RH during the day and 18 °C at night, with a 16-hour day and 8-hour night

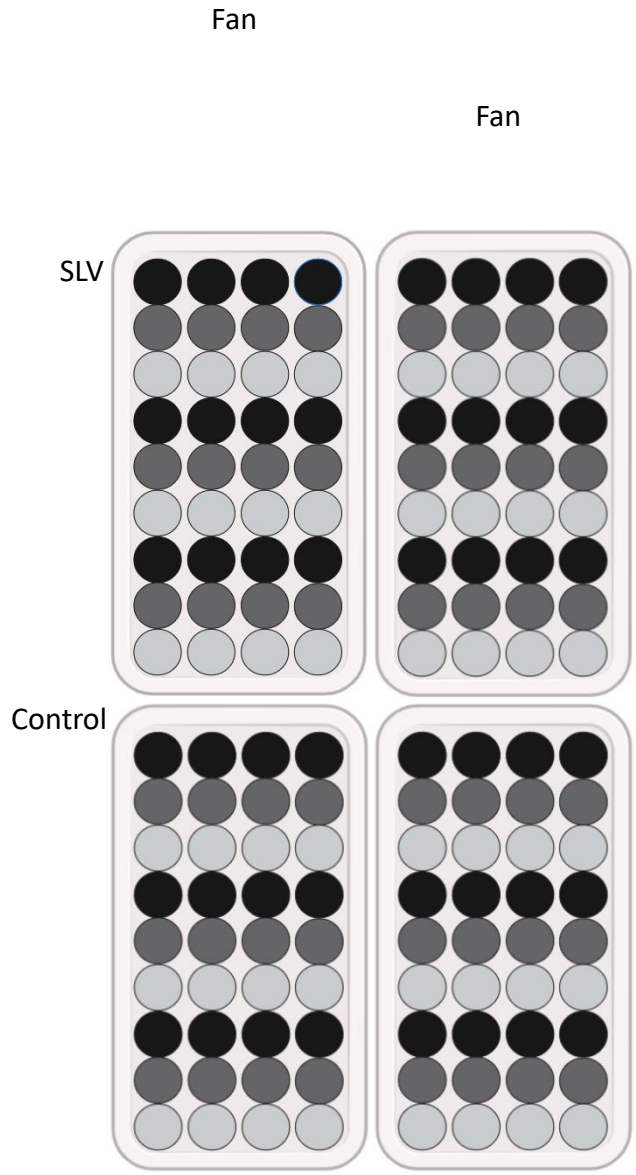
light cycle. The plants were grown under natural light conditions. The plants were watered every three days using drip irrigation for three minutes each time.

The experiment consisted of two soil treatments (SLV and control) and three biofertilizer treatments (two biofertilizers and one control) or six unique treatment combinations. Each treatment combination had 24 replicates (soil with and without biofertilizer and control soil with and without SLV soil; see Figure 2.2). One week prior to planting, soil samples were taken from the SLV at a depth of 15.24 cm using a spade and stored in five-gallon buckets. The soil was collected from a field where tubers exhibited severe symptoms of corky ringspot disease during the previous season (i.e., 2022). The control soil was composed of fine-medium vermiculite (American Clay Works., Denver, CO), Lambert black peat moss (American Clay Works., Denver, CO), and mason sand (Pioneer Sand Co., Windsor, CO) at a 1:1:1 ratio. One-gallon pots (15.24 x 17.78 cm) were filled with soil substrates. Neither soil type was sterilized to maintain the integrity of the natural nematode fauna in the SLV soil compared to the control soil. The SLV pots were filled with a 1:1 dilution of SLV and control soil.

The experiment setup occurred over three consecutive days, from May 16 to May 18, 2023 (Replicate groups 1-3) because our research team did not have sufficient resources to complete the setup in a single day. Tubers were halved, and one-half was planted in the soil at a depth of 10.16-15.24 cm in the center of each pot and drenched with 5 ml of biofertilizer or water. Each pot's top 2.54-7.62 cm layer of soil was mixed with 22.18 ml of Osmocote® Plant Food smart-release flower and vegetable. Pots were separated on four tables in the UNCO greenhouse by soil type (control and SLV). The positions of the pots on the two sets of tables were changed arbitrarily every three weeks, with the restriction that pot positions were limited to tables with the same soil type.

Figure 2.2

Greenhouse Experiment Setup



Note. The experiment was setup over three days (noted as 'replicate groups'), each containing replicated treatments. 1) water, control soil (-biofertilizer/ -SVL); 2) AM fungi, control soil (+AM fungi/ -SVL); 3) *Beauveria bassiana*, control soil (+*B. bassiana*/ -SVL; 4) water, San Luis Valley soil (-biofertilizer/ +SVL; 5) AM fungi, San Luis Valley soil (+AM fungi/ +SLV); and 6) *Beauveria bassiana*, San Luis Valley soil (+*B. bassiana*/ +SVL). There were two fans placed on the West side of the greenhouse. Every pot contained a single plant, and the three different colors of circles in this figure represent the three different biofertilizer treatments. Two tables held pots with SLV soil, and two tables held pots with control soil. Each treatment combination consisted of 24 pots distributed across two tables. The positions of the pots were arbitrarily changed every three weeks.

Biofertilizers

In this study, we applied 5 ml of each biofertilizer or water to the soil and a half seed of every pot. The biofertilizers used in the study were MycoApply® Endothrive (Mycorrhizal Applications., Grant Pass, OR) and Terregena®SPE-120 (Terregena Inc., Raleigh, NC). MycoApply® Endothrive is composed of a liquid solution that contains the AM fungi species *Glomus intraradices, Glomus mosseae, Glomus aggregatum,* and *Glomus etunicatum,* each at 381 propagules/ml. MycoApply® Endothrive was diluted with water to the company's recommended application concentration of 37.85 ml/l. Terregena®SPE-120 is composed of *Beauveria bassiana* at 5.6 x 10^3 propagules /ml. BA was diluted with water to the commercially recommended concentration of 0.047 ml/l. The plant tissues and soil were collected from each pot between August 25 and August 27, 2023. Pots planted on the first day of sowing were the first group harvested on the first day of harvest, and so on.

Plant Measurements

Stem length was measured weekly from germination to harvest. The plant tubers, roots, shoots, and soil in the treatments were harvested in August 2023. The shoots were dried at room temperature in the greenhouse for one week and weighed using a scale to determine the shoot dry mass of each plant. The number of tubers per plant was counted, and the yield of each plant was measured by weighing the mass of all the tubers in each pot on a scale. The diameter of tubers determines their value when taken to market. The diameter of transected tubers was measured using a ruler to calculate the diameter at harvest, five ranges of tuber diameter were generated using the quartiles of the dataset (Boussageon et al., 2023). A subsample of each root section was collected in 50 ml vials in 50% ethanol and stored in the University of Northern Colorado's cold room at 4°C before staining.

Plant Root Hyphal Colonization

Root samples were stored in 50% (v/v) ethanol at harvest. A 1-3 g root slice sample was randomly collected from 3 plants within each treatment combination. Clearing and staining were performed according to the protocol by Vierheilig et al. (1998). Root section samples were cleared for 24 hrs in 10% (w/v) KOH at 37 °C. Cleared sections were rinsed in deionized water and soaked in 5% (v/v) glacial acetic acid for 10 min. Sections were then stained with 5% (v/v) Shaeffer Black ink (prepared in 5% (v/v) glacial acetic acid). The roots were rinsed with deionized water after the ink was removed and checked for staining of fungal hyphal structures in the root tissues on a dissecting scope (Leica DMC 4500; Deb et al., 2022; McGonigle et al., 1990; Vierheilig et al., 1998).

Nematode Soil Density

After collecting soil from each pot post-harvest, it was transferred to Colorado State University and stored at 4 °C within three days of being harvested. The nematode extraction and evaluation were carried out within a week of storage. The presence of nematodes was determined by calculating the number of nematodes per kilogram of dry soil. A subsample of 50 g from each pot was dried for 48 hours at 105 °C in an incubator and subsequently weighed to measure soil moisture (Ankrom et al., 2022). The nematodes in 100 g samples from the pots containing SLV soil were extracted using the Baermann funnel method modified by Diana Wall in 2004 and stored in 10% (v/v) formalin (Ankrom et al., 2022; Baermann, 1917; Flegg & Hooper, 1970). All nematodes from each pot in these 5 ml samples were quantified in counting dishes using an inverted microscope (Olympus CK30) at x200 magnification.

Statistical Analysis

Statistical analyses were performed in R studio for Mac (R Core Team, 2022). The replicate group, i.e., day of planting/harvest, was explored in preliminary analyses but did not have a statistically significant effect, so it was not included in the final models. The normal distribution of the data was assessed using the Shapiro-Wilk and skewness tests with a significance level of $p > .05$.

Potato tuber mass did not exhibit a significantly positive distribution, so it was not transformed. The data related to tuber counts and dry shoot mass exhibited a positive distribution and were subsequently transformed using the square root method. The length of stems and the density of nematodes in the soil demonstrated a positive skew, requiring a log transformation.

A two-way ANOVA was employed to examine the influence of soil and treatment types on plant growth parameters. Additionally, a one-way ANOVA was utilized to scrutinize the effects of treatments on nematode density. Upon completing this analysis, a Tukey's post hoc test was performed at a significance level of $p > .05$ to investigate the differences among treatment types.

CHAPTER III

RESULTS

Objective 1: Effect of Biofertilizer Application on Plant Growth Parameters

The research hypothesized that the application of biofertilizers would improve shoot mass, height, tuber number, yield, and diameter over water alone. The pilot work further suggested that MycoApply Endothrive (AM) might have greater plant growth measurements. After conducting a comparative analysis of the two soil types, it was observed that the San Luis Valley (SLV) soil had a significantly negative impact on yield, tuber count, shoot dry mass, and shoot height when compared to the control soil (see Table 3.1, Figure 3.1). On average, the potato plants grown in SLV soil exhibited less tuber mass, fewer tubers, lower dry shoot mass, and shorter shoot height compared to the control soil plants.

Table 3.1

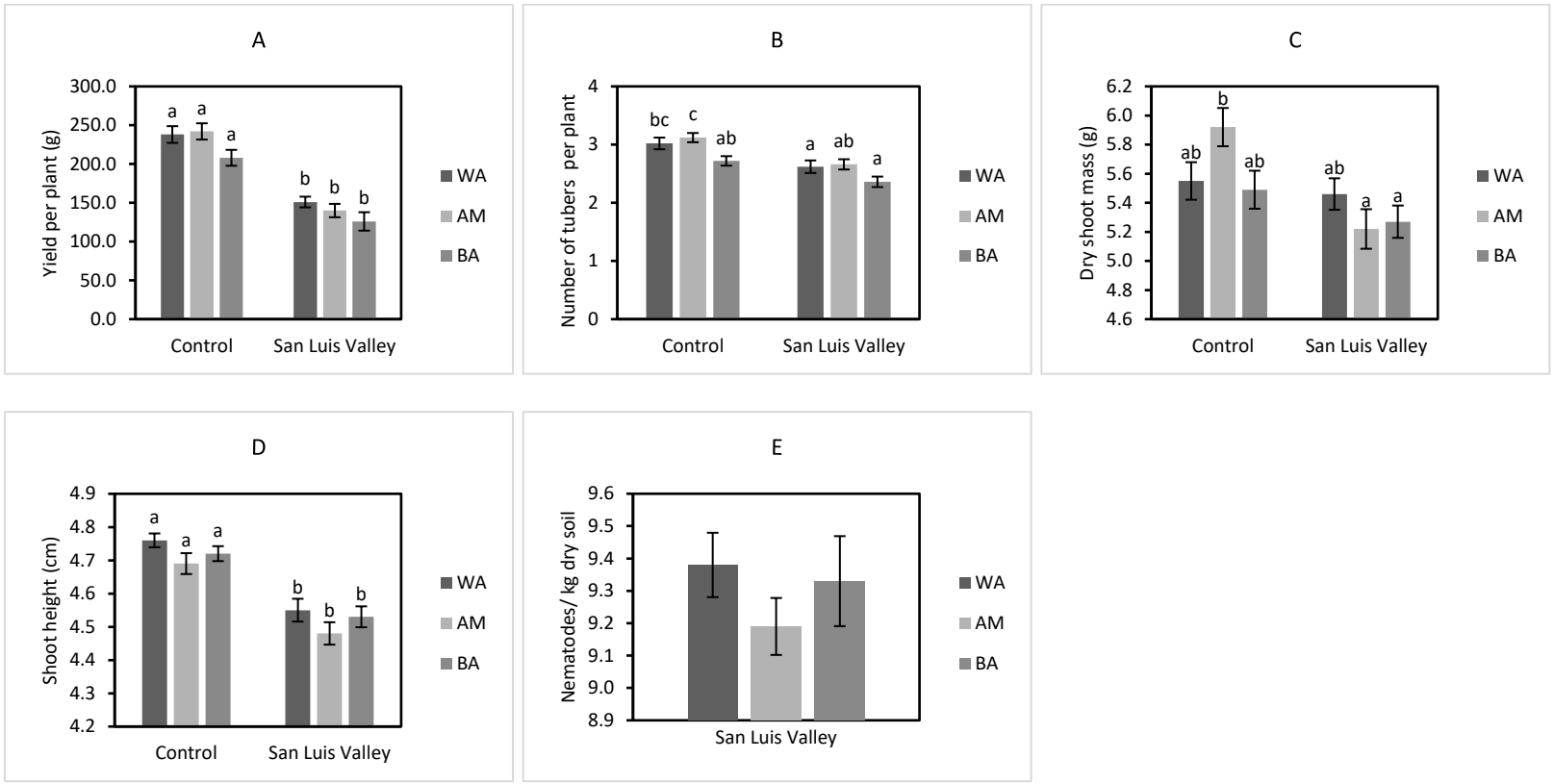
Soil and Treatment as Interacting Main Effects in Linear Models of Growth Variables

		Yield		Tubers/plant		Shoot dry mass		Shoot height		Nematode density	
Factor	DF	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Biofertilizer and Soil Comparison											
Soil	1	124.198	**<0.001**	29.41	**<0.001**	10.747	**0.001**	67.026	**<0.001**		
Treatment	2	4.56	**0.012**	8.184	**<0.001**	1.222	0.298	2.734	0.069	0.807	0.45
Soil*Treatment	2	0.533	0.588	0.153	0.859	3.235	**0.042**	0.036	0.965		

Note. Two-factor ANOVA between soil and biofertilizer treatment predicting tuber yield, tubers per plant, dry shoot mass, and shoot height. F statistic (F), *p*-value (p), $N = 144$.

Figure 3.1

Biofertilizers and Soil Impacts on Growth Parameters and Nematode Density



Note. Impact of biofertilizer treatments and soil type on potato (*Solanum tuberosum* cv. Masquerade) yield (A), tuber number (B), shoot mass (C), shoot height (D), and nematode soil density (E). WA = water, AM = AM fungi, and BA = *Beauveria bassiana*. When an ANOVA showed statistical significance between treatments ($p < 0.05$), a Tukey's post hoc test was performed. Letters represent significant differences among biofertilizer treatments for each soil independently according to Tukey's post hoc test; values marked by the same letter are not significantly different at $p < 0.05$; Values with no marking were not significantly different at $p < 0.05$ in the ANOVA. Values represent means ± SE, N = 144.

The application of biofertilizers during planting did not significantly impact any variables except tuber number (Table 3.1, Figure 3.1). A two-way ANOVA revealed a significant treatment effect where the water treatment in the control soil (-biofertilizer/ -SLV) significantly outperformed that in the SLV soil (-biofertilizer/ +SLV), resulting in increased tuber count, yield, and shoot height (Table 3.1). Significant differences in tuber numbers were observed between the biofertilizers applied within the control soil (-SLV). Specifically, the *Beauveria bassiana* (BA) treatments (+*B. bassiana*/ -SLV) resulted in significantly fewer tubers per plant than the AM fungi (+AM fungi/ -SLV) treatments in the control soil but not the water treatment ($p = .028$, Figure 3.1 B).

It was found that the interaction between soil type and treatment was not statistically significant for yield, tuber count, and shoot height. However, shoot dry mass showed a minor statistically significant difference, with the pairwise analysis revealing no significant differences between biofertilizer treatments (Table 3.1, Figure 3.1 C). When tubers were separated by size class, it was found that biofertilizer treatment and soil type significantly influenced tuber diameter ranges' frequency (see Table 3.2, Figure3.2).

Table 3.2

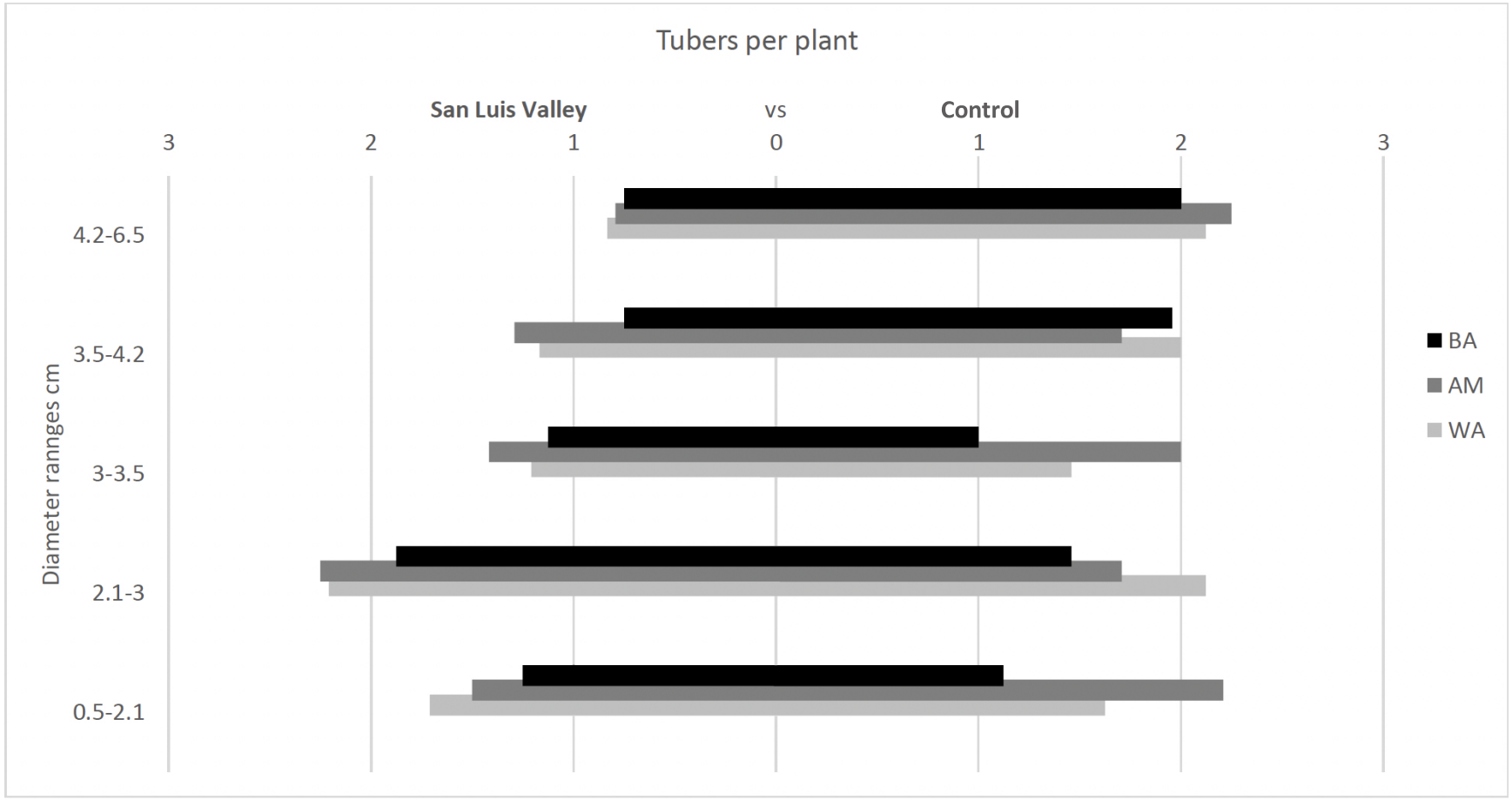
Tuber Diameter Range as Interacting Main Effect with Soil and Treatment on Tuber Frequency

Factor	*DF*	*F*	*p*
Diameter, biofertilizer, and soil comparison			
Diameter Range	4	3.969	**0.003**
Soil	1	19.725	**<0.001**
Treatment	2	5.655	**0.012**
Soil*Treatment	2	0.234	0.791
Diameter*Treatment	8	0.651	0.735
Diameter*Soil	4	8.484	**<0.001**
Diameter*Soil*Treatment	8	0.904	0.512

Note. Three-factor ANOVA between tuber diameter soil and treatment for tuber frequency. F statistic (F), *p*-value (p), $N = 720$.

Figure 3.2

Treatments Effect on Numbers of Tubers per Plant from Five Tuber Diameter Size Ranges



Note. Distribution of tubers per diameter (cm) range based on biofertilizer treatments. Tuber ranges were determined using the dataset's quantiles. A three-way ANOVA was performed to compare biofertilizer treatments within each diameter range. Values represent means, N = 144. WA = water, AM = AM fungi, BA = *Beauveria bassiana*.

Objective 2: Biofertilizer Application on Soil Nematode Presence

This research also hypothesized that biofertilizer application would negatively impact nematode soil density. However, biofertilizer application had no significant effect on the density of nematodes in the soil (Figure 3.1 E). Potato root colonization by AM fungi and *B. bassiana* was not detected in the present study.

CHAPTER IV

DISCUSSION AND CONCLUSION

This study explored the effectiveness of two fungal biofertilizers in impacting their growth attributes, which likely resulted from boosting the potato plant's resistance to soil-plant parasitic nematodes in a controlled greenhouse setting. The study tested three hypotheses: first, that using biofertilizers would enhance potato plant growth by increasing shoot mass and height, as well as the number, yield, and diameter of the tubers; second, that applying biofertilizers would have a negative impact on the density of soil nematodes; and third, that using *B. bassiana* biofertilizer would have the most significant negative impact on nematode density in the soil. Two fungal biofertilizers were investigated: one composed of multiple arbuscular mycorrhizal fungi and another containing an endophytic strain of *Beauveria bassiana*. The soil used in this study was control soil, and SLV soil was sampled from fields where potatoes with corky ringspot disease were collected.

The study revealed that the growth parameters of potatoes were significantly influenced by the type of soil in which they were cultivated in the greenhouse. Specifically, potatoes grown in the San Luis Valley soil showed a reduced yield and shoot height than those grown in the control soil (Figure 3.1). The experiment involved two distinct types of soil, an unsterilized control and soil taken directly from a field where plant parasitic nematode activity was observed to impact tubers. This difference in soil types likely resulted in differences in nutrient content, microbial diversity, and physical properties, all of which are crucial factors influencing plant health and growth (Daniels, 2016). Despite the application of chemical fertilizer to the top of the

soil of the pots for both soil types, the San Luis Valley soil, known for its history of crop cultivation and irrigation, exhibited lower yields (Daniels, 2016; Ronnenberg & Wesche, 2011). This could be attributed to irrigation practices affecting soil composition, leading to the leaching of essential nutrients such as nitrogen and phosphorus. This erosion process can decrease the availability of vital nutrients to crops, resulting in lower yields unless the loss is counteracted with the addition of chemical fertilizers (Daniels, 2016).

Additionally, it is likely that the microbial diversity of the two soils varied significantly. The soil microbial community plays a crucial role in nutrient cycling and disease suppression and is directly influenced by agricultural practices (Daniels, 2016). The long history of crop cultivation and irrigation in the San Luis Valley soil might have led to a unique microbial community structure, which could have affected the growth of the potatoes. Furthermore, the San Luis Valley soil was selected because it contained nematodes that transmitted TRV, causing the symptoms of corky ringspot disease in the harvested potatoes. It was expected that the presence of virus-transmitting plant parasitic nematodes would result in poorer potato growth in the San Luis Valley soil (Daniels, 2016).

Addressing the first hypothesis, we observed that biofertilizers significantly influenced certain growth parameters of potatoes, most notably the number of tubers per plant (Table 3.3 and Figure 3.1 B). Compared to the treatment involving AM fungi, the potatoes treated with *Beauveria bassiana* produced the least number of tubers among the control soil treatments (Table 3.1, Figure 3.1 B). However, we found that biofertilizers did not impact any other growth parameters of our samples differently than the water controls nor compared to one another.

While *B. bassiana* has been recognized as an effective agent for controlling insect pests, its potential in managing plant parasitic nematodes in potatoes remains relatively unexplored

(Ownley et al., 2008; Wei et al., 2020; Zhang et al., 2023). Interestingly, *B. bassiana* has demonstrated a capacity to enhance the health and growth of crops unaffected by pests or pathogens, such as corn (*Zea maize;* Zhang et al., 2023). However, its efficacy appears to be crop-specific, as it has been found to be ineffective in improving the yield of crops like red onion (*Allium ascalonicum* L.;Alfiani et al., 2021).

B. bassiana has also been observed to have a detrimental effect on potato yield when it forms an endophytic relationship with the plant (Mwaura et al., 2017). This negative impact is exacerbated in the presence of plant parasitic nematodes, as the fungus attracts them to the potato tubers, causing further damage to the crop (Mwaura et al., 2017). In the context of this study, particularly in the control soil, *B. bassiana* was observed to impact the tuber number (Table 3.1, Figure 3.1 B) slightly negatively. This is potentially due to the fungus utilizing significant resources from its symbiotic relationship with the plants while providing minimal benefits in return. This finding underscores the need for further research into the complex interactions between *B. bassiana*, potatoes, and plant parasitic nematodes.

The first hypothesis also addresses whether biofertilization would impact the average diameters of tubers. This question was asked because a study by Boussageon et al. (2023) found that AM fungi colonization can homogenize the diameters of potato tubers (Boussageon et al., 2023).

The limited impact of biofertilizers on potato yield observed in this study may be attributed to the short cultivation period. The potatoes were grown in a greenhouse for three months from May to August, whereas cultivation periods can span up to four months, providing ample time for growth and tuber development (Boussageon et al., 2023; Kunkel et al., 1950). Future studies allowing for an eight-month growth period may reveal more pronounced

differences between biofertilizer treatment groups. Interestingly, this study found that nematode soil density remained unaffected by a single application of biofertilizers.

Future research could provide valuable insights by examining the presence of stubby-root nematodes and the transmission success of the tobacco rattle virus, particularly in San Luis Valley soils, and their response to AM fungi and *B. bassiana* biofertilizers. Further investigation into the impact of fungal biofertilizers on the density of stubby-root nematodes and their efficacy in transmitting the tobacco rattle virus would also be of interest. The presence of SRN could be determined by extracting nematodes from the soil and performing PCR (Huang et al., 2018). As CRS disease symptoms were not visually observed in the tubers of potatoes grown in the San Luis Valley soil potatoes, the successful transmission of TRV could be confirmed by extracting RNA and performing RT-qPCR in future studies (Mumford et al., 2000).

A challenge encountered in this study was the inconclusive evidence of fungal colonization of the potatoes. Despite adhering to the recommended dilution concentrations provided by the manufacturers, the concentration of *B. bassiana* (5.6x10^3 propagules/ml diluted to 2.63×10^{-2} propagules/ml) applied in this study was lower compared to other studies that applied it at a rate of 5×10^{7} conidia/ml (Mwaura et al., 2017). Similarly, the concentration of the AM fungi in the biofertilizer used was lower than that used in other studies. This study applied the AM fungi biofertilizer at a dilution of 5.77×10^{1} propagules/ml, while other studies have applied solutions at much higher concentrations of 1×10^{6} propagules/ml (Schoenherr et al., 2019; Song et al., 2015). In order to determine the most effective concentrations of these biofertilizers for practical use, further research is needed. It is worth noting that the limited colonization observed might be due to the small sample size used in this study, which might not have been sufficient to detect colonization accurately. This emphasized the importance of

thoughtful experimental design and adequate sample sizes in future studies. Future studies could consider collecting more expanded root samples and utilizing genetic detection methods to accurately identify the presence of root-associated fungi and plant parasitic nematodes (Castrillo et al., 2003; Huang et al., 2018; Lee et al., 2008; Mumford et al., 2000).

In conclusion, this study underscored the complex interplay between soil microbes, introduced plant growth-promoting microbes, and potato growth. It highlighted the potential of biofertilizers, specifically *Beauveria bassiana* and AM fungi, in influencing potato growth parameters, albeit with varying degrees of success. The findings suggested that while biofertilizers could impact certain growth parameters, their effectiveness might be contingent on factors such as soil composition, cultivation period, and the presence of plant parasitic nematodes. The study also emphasized the need for further research to optimize biofertilizer concentrations and frequency of application and explore their interactions with different soil types and nematodes. Ultimately, this research contributed valuable insights into sustainable agriculture, paving the way for a more nuanced understanding and utilization of biofertilizers in crop cultivation.

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