ARBUSCULAR MYCORRHIZAL FUNGI (AM FUNGI) REDUCES *ASCOCHYTA* BLIGHT (*ASCOCHYTA RABIEI*) SEVERITY IN CDC LEADER CHICKPEA (*CICER ARIETINUM*)

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DEDICATION

This thesis represents the culmination of 14 years of education – from upgrading high school courses to obtaining my undergrad degree, and finally my master's degree. This also represents a large part of my children's lives who have been so supportive and patient throughout the course of my studies. I owe them immense gratitude for their sacrifices. I hope to have instilled in them a strong work ethic, perseverance, and an example of setting and obtaining goals. It was never easy, but it was worth it.

And to Mika dog for keeping my feet warm.

"If you get tired, learn to rest, not to quit." ~ Banksy

ABSTRACT

Cicer arietinum (chickpea) production is limited by the pathogenic fungus *Ascochyta rabiei*, causal agent of *Ascochyta* blight (AB), that has evolved insensitivity against the FRAC Group 11 fungicides. Novel methods of pathogen control as part of an integrated pest management plan are required. Arbuscular mycorrhizal fungi (AM fungi) form a symbiotic relationship with the roots of 80% of land plants that exchange soil-derived nutrients for photosynthetic carbon and have been shown to reduce disease severity in several crops. The experiments described herein explored the potential of AM fungi inoculation and common mycorrhizal networks (CMN) to support chickpea plant health when challenged with AB in a greenhouse. Employing an H-pot design and hyphal size exclusion mesh, chickpeas were challenged with *A. rabiei*, and visual disease ratings recorded. The results showed that AM fungi inoculation and the CMN significantly decrease AB disease severity in chickpea. This research is an important preliminary step towards finding sustainable, integrated pest management methods of managing AB.

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LIST OF ABBREVIATIONS

AAFC	Agriculture & Agri-Food Canada
AB	*Ascochyta* blight
AM	Arbuscular mycorrhizal
ATP	Adenosine triphosphate
Cd	Cadmium
CDC	Crop Development Centre
CMN	Common mycorrhizal network
CN-	Cyanide
dH_2O	Distilled water
ECM	Extracellular material
ET	Ethylene
EU	European Union
GM	Genetically modified
IPM	Integrated pest management
ISR	Induced systemic resistance
JA	Jasmonic acid
K	Potassium
LER	Land equivalency ratio
MAS	Marker assisted selection
Mg	Magnesium
MIR	Mycorrhizal induced resistance
Mn	Manganese
MoA	Mode of action
N	Nitrogen
OM	Organic matter
P	Phosphorus
Pi	Inorganic phosphorus
PAMP	Pathogen associated molecular pattern
PAA	Plant affected area
PDA	Potato dextrose agar
PGPM	Plant growth promoting microbes
pH	Potential of hydrogen
PRR	Pattern recognition receptors
PSB	Phosphate solubilizing bacteria
PTI	PAMP triggered immunity
QoI	Quinone outside inhibitor
QTL	Quantitative trait loci
RCBD	Randomized complete block design
ROS	Reactive oxygen species
S	Sulphur
SA	Salicylic acid
SOC	Soil organic carbon
Zn	Zinc

CHAPTER 1: INTRODUCTION

1.1 *Cicer arietinum* (Chickpeas)

Since 7500 BCE, *Cicer arietinum* (chickpea, aka garbanzo beans) has been the only species out of 46 in the genus *Cicer* cultivated by humans (Gayacharan *et al.,* 2020; Varshney *et al.,* 2019). Chickpeas were imported to North America from Central Asia and East Africa and are now one of the most important pulse crops in the world (Varshney *et al.,* 2019). The main producer of chickpeas is India, while smaller producers include Iran, Mexico, and Canada (Jukanti *et al.,* 2012). In 2021, Canada produced 171 thousand tonnes of chickpeas (Food and Agriculture Organization of the United Nations, 2021). Chickpea crops have a high yield potential; however, increasing drought (Varshney *et al.,* 2014) and disease (Maphosa *et al.,* 2020) hamper higher productivity (Chongo *et al.,* 2004).

As the human population grows, chickpea is becoming an attractive, healthy, and environmentally friendly food option (Boukid, 2021; Kaur & Prasad, 2021). The nutritional profile of chickpea includes proteins, carbohydrates, essential amino acids, vitamins, minerals, starches, dietary fibre, glucose, sucrose, and most importantly, the unsaturated fatty acids linoleic acid and oleic acid (Jukanti *et al.,* 2012; Kakaei *et al.,* 2024). As part of a balanced diet, chickpeas can have beneficial effects on human diseases including diabetes and cancer, while supporting heart and digestive health (Jukanti *et al.,* 2012; Kakaei *et al.,* 2024).

Chickpea plants are diploid, self-pollinators that are highly indeterminate, especially when the weather is cool and wet (Saskatchewan Pulse Growers, 2024). Commercially grown classes of chickpea include both Kabuli and desi seeds (Kaur & Prasad, 2021). Kabuli are large, round, and pale in colour, producing white flowers (Kaur & Prasad, 2021). Desi (aka black chickpeas) seeds are smaller, more angled, darkly coloured, and produce

purple flowers (Kaur & Prasad, 2021). Seed maturity is reached between 110-130 days with two to three seeds per pod (Saskatchewan Pulse Growers, 2024).

1.1.1 Chickpea contribution to soil health

In terms of soil health, chickpea root exudates are beneficial in general as both phosphorus (P) and nitrogen (N) are mobilized leaving inorganic phosphorus (Pi) in the soil for successive crops (Fletcher *et al.,* 2016). Nitrogen acquisition by chickpeas is accomplished by making a symbiotic relationship with soil microbes: (a) rhizobium bacteria that create nodules on the roots and fix atmospheric nitrogen; and (b) arbuscular mycorrhizal (AM fungi) that scavenge the far reaches of the soil beyond the rhizosphere, transporting Pi to the roots (Mohammadi *et al.,* 2011). As a result of N fixation, protons are exuded from their roots into the soil matrix, altering the pH, helping to mobilize potassium (K) and magnesium (Mg) (Fletcher *et al.,* 2016). In northern climates, chickpeas' ability to fix atmospheric N has been found to be 135 kg/ha (Grains Research & Development Corporation, 2013). Soils have also been found to have a higher total soil organic carbon (SOC) content, organic matter (OM), and plant growth nutrients when chickpeas are grown (Mukherjee *et al.,* 2021). This can be partially attributed to the chickpea endophytic bacteria, referred to as plant growth promoting microorganisms (PGPM), that improve soil health and improve chickpea productivity with increased plant health, dry biomass, height and yield due to increases in chlorophyll a, b and total chlorophyll (Mukherjee *et al.,* 2021).

1.1.2 Chickpea genetic resistance to disease

Chickpea resistance is quantitative, controlled by major genes and minor modifiers (Singh *et al.,* 2023). At present, one of the highest resistant varieties is the Kabuli chickpea CDC Leader possessing horizontal, polygenic, partial resistance (Ilyas *et al.,* 2022; van der Plank, 1966). The genetic resistance background of the plant is important since monogenic

(one gene) is less resistant to pathogen evolution than polygenic (multiple genes) resistance (Martins *et al.,* 2020). It is easier for the pathogen to obtain mutations that confer resistance to one set of genes versus multiple sets. Since the chickpea gene pool is limited with regards to quantitative trait loci (QTL), induced systemic resistance (ISR) against fungal pathogens may be important in development of chickpea durable resistance in the future (Nitnavare *et al.,* 2022). Even when appropriate genes are located that provide enough durable resistance alternative disease control methods are required to ensure this genetic resistance persists (Nitnavare *et al.,* 2022). The issues for breeding programs when attempting to identify new genes include varying resistance by chickpea genotype, whether that resistance is mono or polygenic, and the number of genes involved (Cho & Muehlbauer, 2004).

The biggest threat to chickpea production is the fungal pathogen *Ascochyta rabiei*, causing *Ascochyta* blight (AB). The high diversity of *A. rabiei* pathotypes on the Canadian prairies poses a threat for overcoming any resistance instilled by successful breeding (Sharma & Ghosh, 2016; Vail & Banniza, 2008). Chromosomes 2 and 4 have been identified as promising targets (Sharma & Ghosh, 2016) for QTL, and hybrid chickpeas have been crossed that revealed three QTLs on chromosomes 2, 3, and 6, and 7 are activated during either early and late AB (Lakmes *et al.,* 2023). Since 2000, many dominant and recessive genes associated with AB resistance have been discovered leading to the conclusion that resistance is most likely due to inheritance of these genes, in addition to additive gene action (Ilyas *et al.,* 2022; Singh & Reddy, 1983). Defensin genes have also been recently identified and characterized in chickpea that provide protection against hemi-biotrophic and necrotrophic fungal pathogens, like *A. rabiei* (Nitnavare *et al.,* 2022). Having antimicrobial activity, these defensins represent a promising new target of crop

defense research. Mechanisms of action include reduction of hyphal growth and inhibition of key fungal enzymes including pectinases that break down plant cell walls (Andam *et al.,* 2020).

1.1.3 Chickpea defenses against fungal pathogens

The first physical barrier encountered by a foliar fungal pathogen like *A. rabiei* is the cuticle film covering the chickpea leaves (Gniwotta *et al.,* 2005). The cuticle of pea plants has been found to contain over 70 compounds mainly composed of alcohols and alkanes (Gniwotta *et al.,* 2005). These compounds are important since within five minutes of spore contact with the leaf, the spore releases an extracellular material (ECM) composed of hydrolytic enzymes that permits adherence to the leaf surface that also starts the process of cuticle digestion for subsurface invasion (Gniwotta *et al.,* 2005; Martins *et al.,* 2020). Chickpea inducible defenses respond to substomatal pathogen recognition receptors (PRRs) that recognize conserved patterns in the fungi (e.g., chitin) (Martins *et al.,* 2020; Mithoe & Menke, 2018). The specific pathogen molecules like chitin that PRRs recognize are called pathogen associated molecular patterns (PAMPs) that trigger signaling cascades within the plant to activate defense response gene transcription known as PAMP-triggered immunity (PTI) (Coram & Pang, 2006b; Martins *et al.,* 2020; Mithoe & Menke, 2018).

1.2 Arbuscular mycorrhizal fungi (AM fungi)

Since the evolution of land plants over 400 million years ago, AM fungi have made an obligate symbiotic relationship with the roots of up to 80% of plants (Selosse *et al.,* 2015). The etiology derives from the Greek "*mycos*" (fungus) and "*rhiza*" (root). This beneficial fungus lies within the Glomeromycota phylum consisting of seven genera composed of over 150 species (Johnson & Gehring, 2007; Walker *et al.,* 2021). Arbuscular mycorrhizal fungi access pores in the soil that are too small for roots, absorb nutrients and transport

them to the plant root in exchange for photosynthetic carbon (Parniske, 2008). Hyphae extend the rhizosphere by up to 10 cm, well beyond the nutrient depletion zone, accessing nutrients that are non-motile (Thompson *et al.,* 2013). Transportation of nutrients occurs via cytoplasmic streaming at a rate of 12.6 cm h^{-1} through the aseptate (no barriers) hyphae (Cox *et al.,* 1980). Nutrients include P, N, manganese (Mn), sulphur (S), and zinc (Zn), among others (Johnson & Gehring, 2007).

1.2.1 Symbiotic association

When an AM fungi spore germinates it extends the branching germination tube through the soil using chemotaxis to locate the plant root through root exudates (Tikhonovich & Provorov, 2007). The spore has enough metabolic energy that if a root is not found within a week it can retract the germination tube and make multiple attempts (Tikhonovich & Provorov, 2007). Once the root is located, the hyphopodium attaches to the epidermis (Giovannetti & Citernesi, 1993) from which the hyphae extend to cross the root cortex and enter the endodermis (Tikhonovich & Provorov, 2007). Arbuscules and vesicles can then be created within each individual root cell (Gutjahr & Parniske, 2013). Arbuscules ('little trees') increase the surface area for nutrient exchange within the apoplastic compartment through the fungal secretion of orthophosphate that permits absorption of molecules (Gutjahr & Parniske, 2013). The arbuscules are dissolved by the plant after three to five days (Gutjahr & Parniske, 2013; Thompson *et al.,* 2013) while new ones are created in neighbouring cells from existing intraradical hyphae (Tikhonovich & Provorov, 2007). The vesicles are the storage organs of AM fungi for lipids and carbon, while spores are created when the fungus receives a signal from the plant that it is senescing (Gutjahr & Parniske, 2013). The spores allow the fungus to survive until it can make a symbiosis with new living roots (Tikhonovich & Provorov, 2007). Mycorrhizal symbiosis has been reported to

increase plant tolerance to abiotic stresses including drought (Bahadur *et al.,* 2019; Chandrasekaran & Paramasivan, 2022; Recchia *et al.,* 2018), heavy metals (Janeeshma & Puthur, 2020; Ma *et al.,* 2019), heat (Begum *et al.,* 2019), salinity (Evelin *et al.,* 2019; Porcel *et al.,* 2012), and any combination of these. In addition, it is well established that AM fungi support the defense responses of crops, termed mycorrhizal induced resistance (MIR) that occurs when a plant is colonized by AM fungi either alone or as part of the common mycorrhizal network (CMN) (Pozo & Azcon-Aguilar, 2007). The presence of the fungi within the roots causes a systemic induction of defenses, mostly in priming of defense phytohormones (Cameron *et al.,* 2013).

1.2.2 Common mycorrhizal networks (CMNs)

A common mycorrhizal network (CMN) is formed when plant root systems from the same or different species are connected by the hyphae of AM fungi (Barto *et al.,* 2012; Figueiredo *et al.,* 2021). A lack of plant specificity means that AM fungal hyphae can fuse through anastomoses, becoming useful for transference of nutrients including N fixed by nodules of legumes, and water during times of drought (Song *et al.,* 2010). Water transport occurs both within and on hyphae in cycles, between the plant while it photosynthesises and down the concentration gradient within the soil itself at night (Barto *et al.,* 2012). Common mycorrhizal networks also provide benefits to the connected plants by way of increased plant establishment and survival, and increased defense responses (Figueiredo *et al.,* 2021; Gorzelak *et al.,* 2015). The CMN is a beneficial conduit since exudates and other communication chemicals are degraded quickly once they reach the soil (Barto *et al.,* 2012), making transference of signals through the soil itself an unlikely process. The CMN can be likened to rhizomes produced by plants like ginger and the Venus flytrap, or runners produced by strawberry plants, in that these also create networks belowground to transfer

water and nutrients between connected plants (Stuefer *et al.,* 2004). These networks have been found to transfer systemic pathogens (Stuefer *et al.,* 2004), so it is likely that the transference of defense signals also occurs. To illustrate the importance of the CMN, in a faba bean and coix (Job's tears – cereal) intercrop, the CMN increased plant biomass due to supplemental nutrient provision, while suppressing weeds such as foxtail, culminating in an overyielding effect (Qiao *et al.,* 2020).

The hyphosphere, a thin layer of water on the hyphae, is an important part of the CMN when consideration is given to its ability to transport beneficial microbes and molecules through the soil and between plant communities, positively altering plant responses to stress while moderating the beneficial soil microbiome (Cabral *et al.,* 2019; Wang *et al.,* 2022; Zhang *et al.,* 2022).

1.2.3 Affect of fungicides on AM fungi

In Europe, fungicides are required to be ineffective against AM fungal propagules in the soil; however, the issue arises when the roots take up the fungicides that subsequently negatively affect the AM fungal symbiosis (Okiobe *et al.,* 2022). Direct effects on AM fungi can include slowing their metabolism so that their growth is limited or prevented, and altering structures so that the symbiosis cannot occur, while indirect effects include altering the host plant in such a way that the symbiosis is negatively affected (Okiobe *et al.,* 2022). Copper based fungicides can accumulate in AM vesicles, reducing the ability of AM fungi to provide stress protection against heavy metals in the soil, and prevention of arbuscule formation causes a decrease in nutrient exchange with the plant (Okiobe *et al.,* 2022). Foliar fungicides are preferred if they are used in the right dose at the right stage of plant development so that application does not result in spillage onto the soil (Hage-Ahmed *et al.,* 2019). Fungicide can be an inhibitor of AM fungi when applied outside of the

recommended application schedule and dosage rates; AM spores are found to survive in soil and recolonize plant roots in the presence of foliar fungicides when utilized appropriately (Hage-Ahmed *et al.,* 2019). Since AB is a bigger threat to chickpea crops than a lack of AM fungi, this information is positive and should be implemented as part of the integrated pest management (IPM) strategy for producers.

1.3 *Linum usitatissimum* (flax)

Linum usitatissimum (flax)*,* the etiology of which means 'very useful', has been utilized for the past 36,000 years (Baley *et al.,* 2021) and is considered an agricultural founder crop (Weiss & Zohary, 2011). Flax is an annual plant characterized by stems that originate from the base of the plant, producing blueish flowers that are self-pollinated forming multiple seeds (Weiss & Zohary, 2011). The root system of flax is composed of a shallow tap root and is well adapted to different growing regions worldwide (Sertse *et al.,* 2019). Although flax roots can reach depths up to 120 cm, this depth only represents up to seven percent of the total root system and is easily outcompeted by other shallow-rooted crops and weeds (Sertse *et al.,* 2019).

Flax stems are used for fibre and the seed as a source of oil (Goyal *et al.,* 2014). World War 2 airframe reinforcement, paints and varnishes, animal feed, clothing and textiles, and house construction all utilize(d) flax (Goyal *et al.,* 2014; Weiss & Zohary, 2011). Flax is also an important source of omega-3 fats, protein for both humans and animals, a few minerals, dietary fibre, and lignans as a source of antioxidants (Goyal *et al.,* 2014). When used as animal feed and in industry, it is called linseed (e.g., linoleum flooring), whereas when used for human consumption it is called flaxseed (Goyal *et al.,* 2014). In 2021, Canada produced 346,000 tonnes of flaxseed (Franz-Warkentin, 2022) and is considered a global leader in the production of flax, representing more than $200 million in Canadian

exports annually (Ryan & Smyth, 2012). The demand for flax is forecasted to increase, attributed to younger generations being more health conscious and utilizing flax as meal and flour (Goyal *et al.,* 2014; Franz-Warkentin, 2022).

1.3.1 Response to disease

Flax is subject to both biotic and abiotic stressors, the worst being *Fusarium* fungal pathogens that cause wilts and root rots (Wojtasik *et al.,* 2015). *Fusarium oxysporum* invades the roots with the aim of blocking vasculature ultimately resulting in plant death (Edirisinghe *et al.,* 2024; Wojtasik *et al.,* 2015). *Fusarium culmorum* increases pH at the site of infection to support enzymatic degradation of host tissue also resulting in plant death (Wojtasik *et al.,* 2015). In addition to *Fusarium* pathogens, *Mycosphaerella linicola* results in flower and capsule loss, girdling of the stem, and premature plant death (Paumier *et al.,* 2020). Defense mechanisms in flax against fungal pathogens include synthesis of polyamines targeted at reinforcing the plant cell walls to inhibit fungal spread with co-production of hydrogen peroxide (Wojtasik *et al.,* 2015) and oxidative burst upregulating genes in the production of callose and abscisic acid (ABA) (Boba *et al.,* 2022). Other mechanisms include production of secondary metabolites such as lignin and defense phytohormones (Wojtasik *et al.,* 2015).

1.3.2 Symbiosis with AM fungi

Flax benefits from a symbiotic relationship with AM fungi in nutritionally decimated soils (Thompson *et al.,* 2013). In soils inoculated with AM fungi, the levels of both Zn and P within flax roots were significantly higher than those in AM fungi deficient soils (Thompson *et al.,* 2013). In fact, when grown in a crop rotation after a non-mycorrhizal crop such as canola, flax seed yields decrease (Saskatchewan Flax Development Commission, 2022). Since flax is sensitive to N and P, these fertilizers may prevent

germination or stunt growth preventing seed set (Saskatchewan Flax Development Commission, 2022), making the AM fungi symbiosis relevant to increased yields.

1.3.3 Trials and tribulations

Triffid flax was a Canadian genetically modified (GM) flax variety FP967 CDC Triffid developed by the University of Saskatchewan's Crop Development Centre in the 1980's (Ryan & Smyth, 2012). The mechanism of modification is transgenic meaning that another organism's DNA has been transferred into the flax genome (Teli & Timko, 2004). The purpose of this development was to provide growers with a seed that was resistant to persistent soil herbicidal residues that may prevent germination, while still offering producers weed management and crop rotation options (Ryan & Smyth, 2012). The European Union (EU) is a major trade partner with Canada and has a zero-tolerance policy for genetically modified (GM) crops so when the seed was approved by both Canadian and American regulatory bodies in 1998, import by European markets halted one year later (Ryan & Smyth, 2012). In response, Canada destroyed Triffid flax and remaining seed by 2001, but in 2009 the EU detected it once again, preventing export from Canada (Ryan & Smyth, 2012). Detection was based on the presence of the NPTII marker indicating transgenic plants due to kanamycin resistance (de Vries & Wackernagel, 1998). The source was farmer-saved seed and pedigrees in CDC Normandy and CDC Mons varieties (Ryan & Smyth, 2012). Over time, Triffid flax has finally become obsolete in the Canadian market (Ryan & Smyth, 2012).

In 2021, levels of cadmium (Cd, a naturally occurring soil metal) uptake by flax has seen new regulations imposed by the EU at 500 mg per tonne (Wozniak, 2022). New varieties of flax are needed but without adoption of GM flax, options for preventing Cd uptake by the plant are limited to competitive interactions in the rhizosphere with the addition of other

metals such as Zn (Wozniak, 2022). In 2023, the EU once again put restrictions on Canadian flax exports, but this time focused on the levels of hydrocyanic acid (150,000 mg per tonne) in crushed flax seed – concerning because of its transformation into cyanide (CN-) during the human digestive process (Pratt, 2023). However, CN- is present in many foods humans ingest (Yip & Yang, 1988) and it is broken down by action of a mitochondrial enzyme, rhodanese, in the liver (Buonvino *et al.,* 2022). The market trend for production has been predicted to decrease by 42% in 2024, but Canada remains optimistic as demand for flax increases in other markets and prices lower (Agriculture & Agri-Food Canada, 2024).

1.4 *Ascochyta rabiei*

The necrotrophic pathogenic fungus, *Ascochyta rabiei* (Pass) Labrousse*,* is the causal agent of ascochyta blight (AB) of chickpea, an ascomycete anamorph (asexual: *A. rabiei*) with a teleomorph stage (sexual: *Didymella rabiei*) (Chongo *et al.,* 2004). Since *A. rabiei* is a heterothallic organism (individuals are different sexes), two different mating types are required to produce ascospores in the sexual stage (Kaiser, 1973; Trapero-Casas *et al.,* 2012; Trapero-Casas & Kaiser, 1992a). These mating types have been studied in Canada and are both present on the prairies providing a potential source of genetic diversity for evolution of the population (Armstrong *et al.,* 2001). There are 14 pathotypes of the teleomorph, *D. rabiei,* in Canada, each causing disease in chickpea, identified by numbers 1-14 (Navas-Cortés *et al.,* 1998). The most common pathotype is #2, whereas #1 is not virulent on any chickpea cultivar (Armstrong *et al.,* 2001; Navas-Cortés *et al.,* 1998). Although there have not been any significant outbreaks of AB in chickpea in Canada recently, Saskatchewan still reported a disease incidence of 3.2% in 2019 and 2020, with a 7.2% decrease in disease-free seed in 2020 (Elmhirst, 2022).

1.4.1 Mode of infection and life cycle

Ascochyta rabiei infects all above-ground parts of the plant with visual signs being necrotic lesions on leaves and stems that eventually spread to buds and pods, causing girdling of the stem in the advanced stages of infection (Harveson *et al.,* 2011). Pycnidia, the infective asexual spores, form diagnostic concentric rings that are visible to the naked eye (Harveson *et al.,* 2011). Seeds can become infected and turn brown and shriveled with cankers (Harveson *et al.,* 2011) reducing yield and producer profits.

The life cycle of *A. rabiei* begins with pycnidium on chickpea residue following one of two reproductive routes: sexual or asexual (Figure 1). During sexual reproduction, ascospores are produced and enter the disease cycle. During asexual reproduction, which is the most common form of infection, conidia are produced. Conidia infect the leaves, stems, and pods of the plant, germinating after 12 hours and penetrating the tissues within 24 hours of contact (Pandey *et al.,* 1987). The appressorium is a structure formed by the fungus to penetrate the leaf cuticle and invade the plant cell walls (Foresto *et al.,* 2023). Four days post-infection, young leaves will become necrotic while hyphae aggregate in the cortical tissue (Pandey *et al.,* 1987). Under cool and wet conditions, the disease progresses into AB where lesions can be seen on stem tissue with pycnidia visible (Pandey *et al.,* 1987). Stems may girdle and break off (Pandey *et al.,* 1987). Once enough of the plant is infected, death and conidial spread occur rapidly throughout the crop.

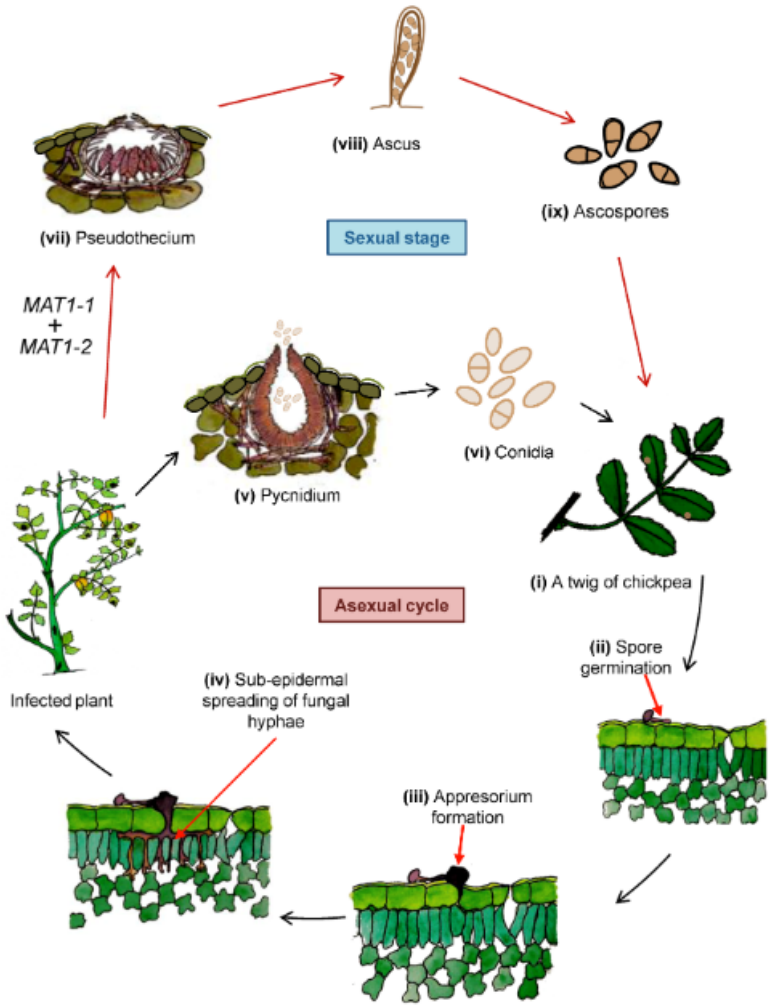


Figure 1. *Ascochyta rabiei* disease cycle depicting both sexual and asexual stages. Image used under Creative Commons Attribution License (Singh *et al.,* 2022).

1.4.2 Sources of inoculum

Inoculum sources include infected seeds and overwintering of pseudothecia on the previous years' crop stubble that eject ascospores to infect new foliage with windborne transmission up to 15 km away (Armstrong *et al.,* 2001; Trapero-Casas *et al.,* 2012). In line with the disease triangle of right pathogen-right host-right environmental conditions, cooler temperatures (<20ºC), high humidity, and upwards of 17 hours of spore contact with the leaf allow germination to occur (Harveson *et al.,* 2011).

1.4.3 Pathogen Control Methods

Current methods to control *A. rabiei* in chickpea crops include fungicide application, improving chickpea genetic resistance, disease-free seed, and crop rotations with non host plants for upwards of four years (Coram & Pang, 2006a; Fanning *et al.,* 2022a; Ilyas *et al.,* 2022; Nitnavare *et al.,* 2022; Reddy & Singh, 1984; Sharma *et al.,* 2011). Even when a moderately resistant cultivar of chickpea is planted, properly timed fungicide application is still required to fully suppress the development of AB (Basandrai *et al.,* 2007). This is the most effective way to prevent disease rather than applying it post-infection (Wilson *et al.,* 2022). However, fungicides are a source of selective pressure for *A. rabiei* that have already become resistant to the mode of action of FRAC Group 11 QoI fungicides (quinone outside inhibitor) (Ahmed *et al.,* 2014; Delgado *et al.,* 2013). Improper use of fungicides also have negative effects on aquatic life, honeybees and related pollinators, soil invertebrates, and the soil microbiome, including the beneficial symbiont, arbuscular mycorrhizal fungi (Baćmaga *et al.,* 2016; Belsky & Joshi, 2020; Gunstone *et al.,* 2021; Zubrod *et al.,* 2019).

1.4.3.1 Fungicides and resistance

The most common method of control for AB is the application of the FRAC Group 11 fungicides (Bartlett *et al.,* 2002). Group 11 fungicides work with a specific mechanism of action (MoA) against the cytochrome bc_1 complex called quinone outside inhibitors (QoI) that prevent adenosine triphosphate production (ATP) in the fungus (Delgado *et al.,* 2013). The first site-specific MoA fungicide was made available just over 50 years ago and since then over 100 plant pathogens have developed insensitivity, with QoI fungicides alone contributing to insensitivity in over 50 species (Corkley *et al.,* 2022). In *A. rabiei* this insensitivity is due to a mutation in the cytochrome b gene (G143A) (Delgado *et al.,* 2013). Given that both mating types of *A. rabiei* are found on the Canadian prairies, it is possible

for the fungus to sexually recombine resulting in novel genetic combinations that could increase aggressiveness and/or insensitivity against other single MoA fungicides. The use of different fungicides with multiple MoA are more effective at controlling disease versus site-specific MoA (Corkley *et al.,* 2022).

Ascochyta blight can also be managed using non-group 11 fungicides, such as those from groups 3 and 7. Group 3 MoA is a demethylation inhibitor that inhibits ergosterol production within the fungus, important for composition of membranes and cells (FRAC, 2024). Group 7 MoA is a succinate dehydrogenase inhibitor that inhibits succinate dehydrogenase production necessary for cellular activity (FRAC, 2024).

There are different mobilities of fungicides that includes systemic or contact. Systemic fungicides are absorbed into the system of the plant where it is transported through the plant vasculature to inhibit fungal growth (UPL, 2024). Contact fungicides act locally where they are sprayed and must come into direct contact with the fungus to kill it (UPL, 2024).

1.4.4 Integrated pest management

Integrated pest management (IPM) is an approach to controlling disease using both chemical and non-chemical methods (Fanning *et al.,* 2022b). In addition to applying fungicides, the use of disease-free seed, crop rotation, resistant cultivars (Fanning *et al.,* 2022b) weather forecasting, and scouting of fields are the best combination for prevention of AB.

1.5 Intercropping

Intercropping is the agricultural practice of planting multiple crops together (Bremer & Greer, 2021; Temesgen *et al.,* 2015) on the same land to be cultivated at the same time (Zhang & Li, 2003). Producers can efficiently utilize soil resources while realizing increased yields through overyielding (Bremer & Greer, 2021), maximizing land use

efficiency calculated through the land equivalency ratio (LER) (Chapagain & Riseman, 2014; Qiao *et al.,* 2016) and reducing the application of inputs (fertilizer, pesticides, machinery) (Naudin *et al.,* 2010). Overyielding may be attributed to the release of chemical root exudates from different plant species mobilizing bound nutrients making them available for current and future crops through this facilitative action (Li *et al.,* 2014) and/or the action of the CMN in increasing plant biomass and productivity (Qiao, 2016). Land equivalent ratio is a method of measuring multi-crop versus monocrop production for the same area of land (Deb & Dutta, 2022). For a successful intercrop, the producer must consider the species and their phenology, harvest times, nutrient requirements, seeding rates, seed separation, and pest management requirements (Dowling *et al.,* 2021). The choice of intercropping type is also important, depending on the desired purpose for the crops. Six types of intercropping include: (a) mixed rows with varying ratios, (b) alternate rows with varying ratios, (c) strip rows, with separate strips of each crop, (d) relay cropping, the delay of planting or early harvest of one crop species, (e) living mulch, undersown with the cash crop, and (f) cover crops, two or more species are used to cover the soil (Dowling *et al.,* 2021).

1.5.1 Intercropping chickpea-flax

As worldwide demand for legumes and oilseeds continues to increase due to market expansion and higher commodity prices (Zentner *et al.,* 2002), producer interest in intercropping these crops has also increased. The most popular legume-oilseed combinations include pea-canola, soybean-sunflower, and chickpea-flax (Dowling *et al.,* 2021; Ehrmann & Ritz, 2014). Although some slight equipment modifications may be required, and potentially an initial increase in labour and time (Bennett & Groten, 2022), the benefits are not to be overlooked.

Chickpea-flax intercropping is an attractive option for growers. Chickpea has a larger root diameter than flax and can either be shallow-or-deep rooting depending on the soil conditions, allowing for more efficient use of water and soil nutrients (Gan *et al.,* 2011). The flax outcompetes the chickpea for soil moisture late in the season, causing the chickpea to mature at the right time (terminal stress) eliminating the need for desiccants (Shaw, 2015). Machinery can also be easily adapted to accommodate both the seeding and harvesting of these seeds (Shaw, 2015).

By intercropping chickpea with a non-host plant such as flax, disease pressure can be reduced (Zhou *et al.,* 2023). A two-year study in Montana reported that AB disease incidence and severity decreased by up to 50% when seeded at a 50% chickpea-50% flax proportion; however, this could also result in lower chickpea yield due to flax competition (Shaw, 2015; Zhou *et al.,* 2023). Land productivity was also greater than one indicating an increase in LER compared to monocropping, by up to 23% (Ostlie & Jacobs, 2022; Zhou *et al.,* 2023). The chickpea variety CDC Leader has moderate resistance to AB and performed the best in the presence of high disease pressure when planted in a 70/30 mixed-row intercrop, out-yielding other varieties tested (Zhou *et al.,* 2023). Therefore, variety, seeding ratios, and intercropping type can be adjusted based on individual producer requirements for increased disease management and higher yields.

1.5.2 Disruptions to disease

By planting flax with chickpea, disease may be disrupted by two mechanical effects: dilution effect (Keesing & Ostfeld, 2021) and barrier effect (Fereres, 2000). Dilution effect can occur by utilizing a non-host crop such as flax that acts to disrupt the transmission of infective spores between susceptible plants (Keesing & Ostfeld, 2021). Although dilution effect has been a controversial topic, a recent meta-analysis confirmed that through

increased biodiversity, such as intercropping, plant-parasitism and plant-herbivory are decreased, and by extension, so are plant-pathogens (Civitello *et al.,* 2015). In barrier effect, a non-host crop can either be planted on the border of the susceptible crop or intercropped as alternating rows, effectively preventing the spread of pathogens and some pests (Fereres, 2000).

Non-mechanical barriers include the CMN through supplementation of nutrients and transference of defense signals (Babikova *et al.,* 2014; Barto *et al.,* 2012; Johnson & Gilbert, 2015), and alteration of the canopy microclimate that reduces the relative humidity that is required for pathogen spore germination, while increasing temperature and light availability that also hamper the pathogen's ability to cause infection (Guo *et al.,* 2021).

OBJECTIVES

The purpose of this study was to conduct preliminary experiments to investigate the potential of AM fungi to reduce AB disease in chickpea, focusing on these specific objectives:

1. To determine if there is a significant decrease in AB disease severity when chickpea is intercropped with flax in the presence and/or absence of a CMN;
2. To explore if the CMN plays a role in decreasing AB disease severity in a chickpea donor-receiver relationship; and
3. To investigate whether AM fungi decreases disease severity in chickpeas in the absence of a CMN challenged with AB while increasing plant height, number of flowers, and biomass.

CHAPTER 2: ARBUSCULAR MYCORRHIZAL FUNGI (AM FUNGI) COMMON MYCORRHIZAL NETWORKS (CMNs) REDUCE ASCOCHYTA BLIGHT DISEASE SEVERITY IN CDC LEADER CHICKPEA

2.1 ABSTRACT

Cicer arietinum (chickpeas) are susceptible to a devastating necrotrophic fungal pathogen, *Ascochyta rabiei*, the causal agent of *Ascochyta* blight. There is a lack of sufficient plant genetic resistance, and the pathogen has evolved insensitivity against FRAC Group 11 fungicides. Arbuscular mycorrhizal fungi (AM fungi) are a symbiotic root penetrating fungus that exchanges soil nutrients for photosynthetic carbon. Arbuscular mycorrhizal fungi form common mycorrhizal networks (CMNs) between plant roots that have been shown to transfer nutrients and potentially defense signals that reduce disease severity in neighbouring plants. Intercropping trials in Canada and the USA have resulted in significantly lower disease severity with chickpeas and flax in mixed and alternating rows. The mechanism for this reduction is currently unknown but may be attributed to the flax or AM fungi. **Purpose:** To test the hypotheses that (A) intercropped chickpea plants connected via a CMN will result in significantly lower disease severity, and (B) the CMN will transfer defense signals from a donor plant (challenged first) to a receiver plant (challenged second) that will result in significantly lower disease severity. **Methods:** Two greenhouse experiments were conducted. In both experiments, an H-pot design allowed for hyphal connection manipulation through size exclusion mesh. Experiment A treatments included two plant pairings (chickpea-chickpea and chickpea-flax), three hyphal manipulation mesh treatments (no mesh, 35µm pores, and 1µm pores), and two disease states (challenged, unchallenged). Visual disease ratings were recorded. Experiment B treatments included one plant pairing (chickpea-chickpea), two hyphal manipulation

treatments (35µm pores and 1µm pores), and one disease state (challenged). Visual disease ratings, plant height, and dry biomass were recorded. **Results:** In Experiment A there was no significant effect of the flax on disease severity, however, there was a significant decrease in disease severity in the mesh that permitted only CMN formation with no root contact. In Experiment B there was no significant effect of the CMN on disease severity in the receiver plants, number of flowers, plant height or dry biomass. These results are an important exploratory step for finding novel, sustainable methods of disease reduction that are required to prevent further pathogen fungicide insensitivity while increasing crop yields and producer profits during outbreak years.

2.2 INTRODUCTION

Chickpea crops worldwide are susceptible to *Ascochyta* blight (AB), a devastating fungal disease caused by *Ascochyta rabiei* (Harveson *et al.,* 2011). *Ascochyta rabiei* produces pycnidia, the asexual fruiting body containing infective spores, that form destructive lesions on stems, leaves, and pods (Harveson *et al.,* 2011) causing up to 100% crop loss during outbreak years (Reddy & Singh, 1990). Although CDC Leader possesses moderate resistance (Andolfo *et al.,* 2016; Meridian Seeds, 2021; Saskatchewan Seed Growers' Association, 2024). There is currently no fully resistant chickpea variety, and any genetic resistance decreases at flowering (Basandrai *et al.,* 2007; Singh & Reddy, 1993; Trapero-Casas & Kaiser, 1992b). *Ascochyta* blight occurs if inoculum is present or blown in by the wind in combination with a cool, moist spring, and anytime during the growing season within five to seven days after a moisture event (high humidity, rain, dew) (Armstrong *et al.,* 2001). Ascospores are the sexual reproductive structures of the fungi that can recombine, providing opportunity to evolve increased aggressiveness against plant resistance and single mode of action fungicides (Sharma *et al.,* 2011).

Land plants and arbuscular mycorrhizal fungi (AM fungi) have engaged in a beneficial symbiotic association for over 400 million years (Selosse *et al.,* 2015). Arbuscular mycorrhizal fungi are obligate biotrophs of plant roots relying on plant-derived carbon in exchange for soil-derived nutrients to complete their lifecycle (Smith & Read, 1997). Arbuscular mycorrhizal fungi create vast mycelial networks that extend the rhizosphere up to 10 cm (Thompson *et al.,* 2013), connect plant roots by CMNs (Barto *et al.,* 2011), and have been proposed to transfer defense signals via their CMN (Barto *et al.,* 2012; Johnson & Gilbert, 2015). For example, the AM fungi network has been proposed to act as a signalling conduit between roots of broad bean plants to warn neighbouring plants when herbivory was occurring by aphids (Gilbert, *et al.,* 2013). Common mycorrhizal networks are also hypothesized to transfer defense signals that prime an uninfected plant's defense system (Fujita *et al.,* 2022; Song *et al.,* 2015). Such was the case for a faster, stronger response by several defense-inducing phytohormones to a bacterial pathogen in tomato plants (Fujita *et al.,* 2022).

Field trials conducted in Canada and the USA indicate that when chickpea and flax are intercropped in mixed or alternating rows, the severity of AB disease is significantly reduced, and typically the yields of both crops are increased (Zhou *et al.,* 2023). Possible mechanisms for this reduction could include dilution effect where the amount of inoculum is decreased per plant (Collins *et al.,* 2020), barrier effect wherein the flax prevents the spread of pycnidia to the chickpeas (Fereres, 2000), a change to the canopy microclimate (Guo *et al.,* 2021) and/or an effect of AM fungi in supporting plant defenses (Dey & Ghosh, 2022).

Two experiments were conducted to investigate whether the CMN plays a role in intercropped chickpea-flax to reduce the severity AB in chickpea, and whether a donor-

receiver chickpea pairing connected via a CMN would decrease disease severity. My hypotheses were: (a) that intercropped chickpea plants connected via a CMN will significantly lower disease severity, and (b) the CMN will transfer defense signals from a donor plant (challenged first) to a receiver plant (challenged second) that will significantly lower disease severity while increasing plant height, number of flowers, and biomass. These experiments are a preliminary step in disease research in chickpeas that show promising results for an integrated pest management (IPM) approach to managing AB.

2.3 METHODS

This study consisted of two experiments: Experiment A investigated the action of the CMN within an intercrop of chickpea and flax; Experiment B investigated the CMN between a donor and receiver chickpea plant challenged one week apart with *A. rabiei*. Methods common to both experiments precede those of each individual experiment.

2.3.1 COMMON TO BOTH EXPERIMENTS

2.3.1.1 Organisms

These experiments were performed using CDC Leader chickpea Kabuli variety, CDC Glas flax variety, *Ascochyta rabiei* strain AR-2019-008, commercial AM fungi inoculant (Rootella® X, Groundwork BioAg, Israel) consisting of 133,600 propagules per gram of *Rhizophagus intraradices* and 33,400 propagules per gram of *Funneliformis mosseae* spores that are known to form associations with chickpea and flax, rhizobium species *Mesorhizobium ciceri* (AgroPlus, Coaldale, AB), and PRO-MIX® organic vegetable potting mix (Premier Tech Home and Garden, Quebec) consisting of MYCOACTIVE PTB297 Technology containing one propagule of *R. intraradices* per gram of mix.

2.3.1.2 Hyphal connection manipulations

Size exclusion mesh was used to manipulate the CMN connection of AM fungi between H-pot plant compartments. The 1 µm mesh treatment completely blocked both root contact and CMN formation; the 35 µm mesh treatment permitted CMN formation; the no mesh treatment permitted both root contact and CMN formation. From this point forward the 1 µm mesh treatment will be referred to as the BLK treatment; the 35 µm mesh treatment will be referred to as the CMN treatment; and the no mesh treatment will be referred to as the NM treatment.

2.3.1.3 H-pot design and assembly

H-pot design (Fig. 2a,b) used a 4”x3” PVC connecting pipe to link two 4”x4” PVC tee-pipes that contained the plants. On either side of the connecting pipe was an identical piece of size-exclusion mesh (Fig. 2c) to manipulate hyphal connections. H-pots were placed on 3” round saucers.

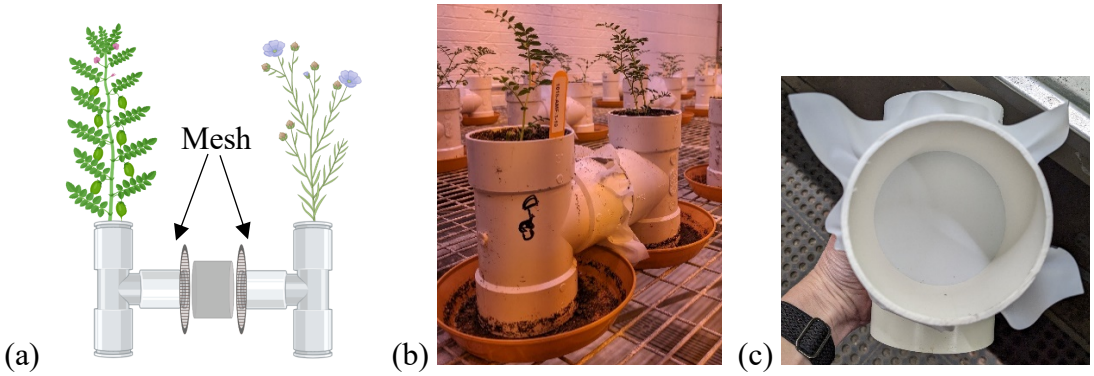


Figure 2. (a) H-pot schematic (created with BioRender.com) modified from Barto *et al.,* 2011 that was used in both experiments. (b) H-pots assembled using 4”x4”x4” PVC tee-pipes and a 4”x3” connector PVC pipe. (c) Size-exclusion mesh treatment to manipulate hyphal connections as seen from the inside of the connector piece.

2.3.1.4 Potting mix and inoculants

PRO-MIX® Premium Organic Vegetable and Herb Mix (Premier Tech, Quebec) purchased from the local Canadian Tire was used as the growing medium in all PVC components (recipe Appendix C). This medium is a peat-based formulation to support AM fungi and plant growth with minute amounts of P fertilizer, too much of which can suppress AM fungi activity (Liu *et al.,* 2021).

Rootella® X was prepared according to the manufacturer's directions: a working solution of 0.5 g xanthan gum was mixed in 1 L of sterilized water, 100 mL of which was used to mix with 1 g of Rootella® X. 1 mL of the inoculant solution, that contained 500 spores, was applied using a 1 mL pipette approximately 2.54 cm into the soil before each seed was planted above this point. Rhizobium inoculant (0.02 g) was applied to the chickpea seed to support root nodule formation for nitrogen fixation (Tavasolee *et al.,* 2011).

2.3.1.5 *Ascochyta rabiei*

Ascochyta rabiei was obtained from Agriculture and Agri-Food Canada (AAFC) in Swift Current in October 2022. This fungus was grown on potato dextrose agar (PDA) plates with 0.1% lactic acid as an antibiotic and stored in the dark at 22°C to encourage spore formation to be used as inoculum (Fig. 3).

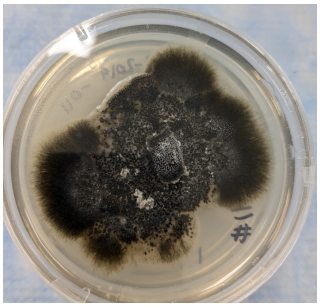


Figure 3. *Ascochyta rabiei* strain AR-2019-008 grown on PDA^{+LA}. (LA: lactic acid)

2.3.1.6 *Ascochyta rabiei* conidial spore suspension and application

The fungal conidial spore suspensions were made fresh on each morning prior to plant challenge. Autoclaved distilled water was used to cover the *A. rabiei* fungal mat in the petri dish and a hockey stick spreader dislodged the spores from the mat into the water that was collected in a sterile glass bottle. A haemocytometer allowed for estimation of spore numbers using a light microscope, being adjusted using sterile water until the spore suspension was appropriate for each experiment (Kaiser, 1973). The final suspension was poured into a sterile spray bottle that released 1mL per spray.

3 mL of conidial spore suspension were sprayed onto the chickpea plants at flowering in a 'dirty' lab to avoid contaminating the greenhouse. To provide a high-humidity environment for disease establishment and prevent contamination of controls, all plants were covered with their own transparent Sun bag (B7026, MilliporeSigma, Canada) that had a Poros stainless steel filter for gas exchange (0.02µm pore size). *Ascochyta rabiei* spores are approximately 3 µm in size (Onosato *et al.,* 2022) so they cannot exit through the Sun bag.

2.3.1.7 Visual disease ratings

Disease ratings were made according to a visual guide as published in Chongo *et al.,* 2004 (Fig. 4). The scale ranges from 0 to 9 and considers the number and size of lesions, the plant affected area (PAA), pycnidia formation, and girdling of the stems (Chongo *et al.,* 2004). At 0, the plant is unaffected and alive; at 2-5, the plant is showing signs of lesions on stems and leaves; at 6-8 the plant has pycnidia and girdling of the stems, while at 9, the plant is fully infected and dead (Chongo *et al.,* 2004).

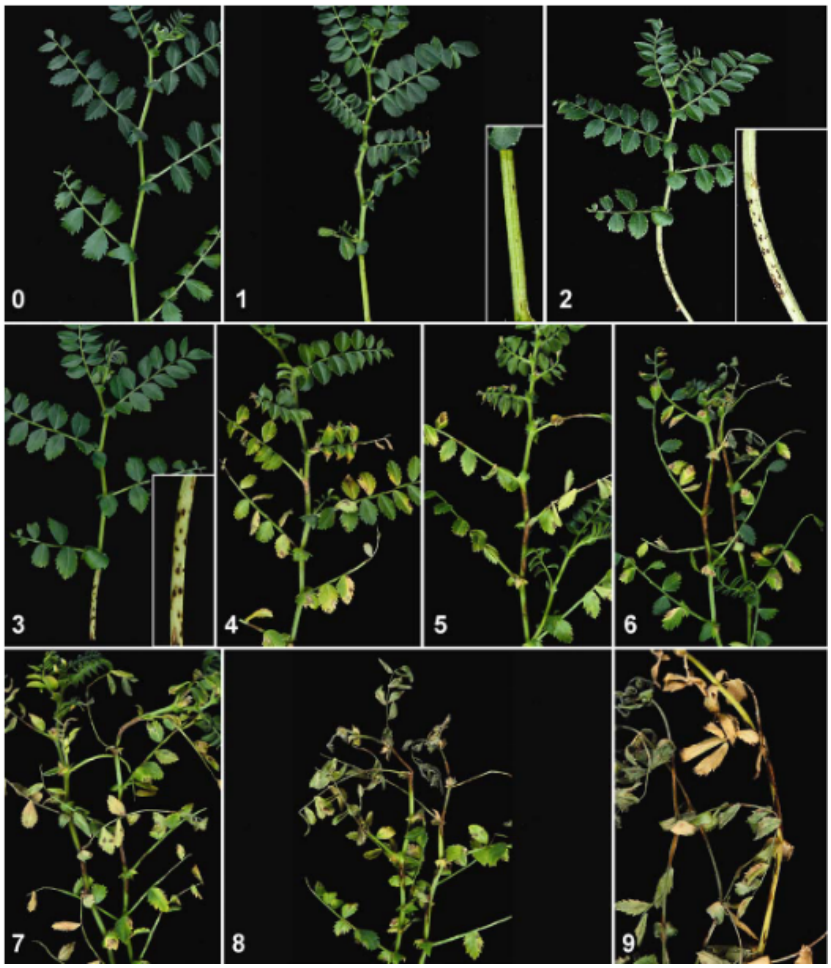


Figure 4. Visual disease rating guide for *Ascochyta* blight (AB) according to Chongo *et al.,* 2004. Disease severity is assigned a number between zero and nine based on plant affected area (PAA) of lesions, stem girdling, pycnidia and plant death.

2.3.1.8 Verification of AM fungal root colonization

To visualize AM fungi structures in flax and chickpea roots, 1 g of roots were washed then cleared of cell walls, cytoplasm, and nuclei by submerging in 10% potassium hydroxide (KOH) for 24 hours in small individually labelled beakers. The roots were rinsed separately in a small sieve in dH_2O for two minutes and transferred to 90ºC Trypan blue (189351000, Acros Organics Fisher-Sci, Ottawa) for three minutes. Roots were removed from the dye and rinsed for another two minutes in dH_2O then destained using acetic acid and milliQ water for at least 20 minutes. Roots were then cut into 20 - 1 cm pieces and

placed on five microscope slides, four per slide. A 50% glycerol solution was placed over each root segment and a microscope cover on each slide. If AM fungal structures were observed at 40x magnification under a light microscope (Fig. 13), results were recorded, and photos taken of significant structures. Structures include hyphae, vesicles, and spores.

2.3.2 EXPERIMENT A: INVESTIGATING A CHICKPEA/FLAX INTERCROP WITH HYPHAL CONNECTION MANIPULATIONS

2.3.2.1 Experimental design

In a greenhouse located at AAFC in Lethbridge, Alberta, a randomized complete block design (RCBD) was implemented for H-pots consisting of two plant treatments (chickpea-chickpea, chickpea-flax), three hyphal connection manipulations (NM, CMN, BLK), and two *Ascochyta rabiei* disease treatments (challenged, unchallenged). Three tables in the greenhouse were divided into 12 blocks of 12 positions (Fig. 5) representing a treatment combination that appeared once in each block. Blocks C and J were empty according to the randomized layout. There were 10 replicates per treatment combination for a total of 120 pots. H-pots were grown with a photoperiod of 12:12 and a temperature of 23ºC daytime, 18ºC night-time, until pathogen challenge when temperature was decreased to a constant 17ºC. The plants were hand-watered when the soil was dry by filling their saucers to ensure all plants received the same amount of water.

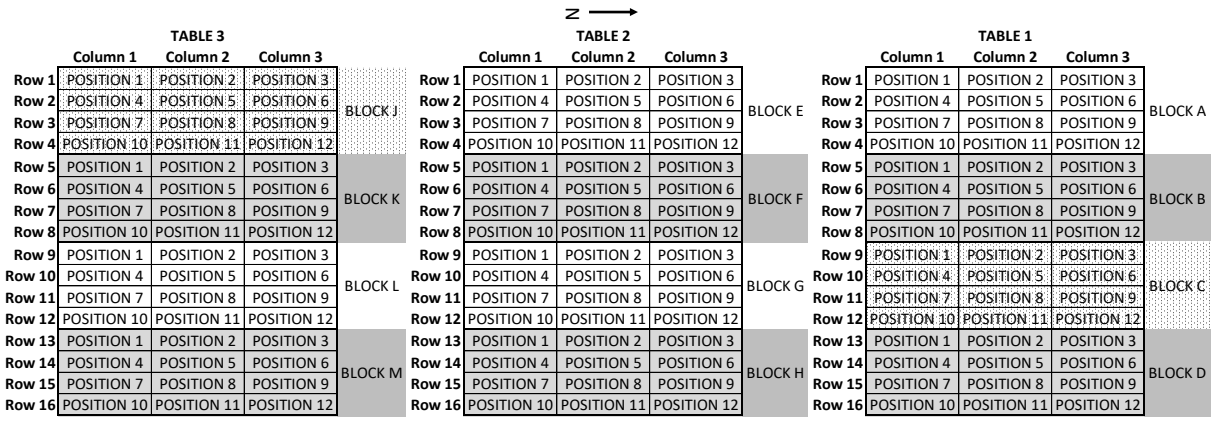


Figure 5. Visualization of the randomized complete block design (RCBD) implemented in the intercrop experiment. Excel was used to randomly assign pots to their blocks and positions with Blocks C and J remaining empty.

2.3.2.2 Seed preparation and germination

215 chickpeas and 60 flax seeds were surfaced sterilized in 5.25% hypochlorite for 10 minutes and rinsed four times in sterilized dH_2O. The chickpeas were germinated in the dark for five days in a petri dish containing two filter papers moistened with the sterilized water. The flax were sterilized immediately before planting without pre-germination of the seeds. Seeds were grown in seedling trays with PRO-MIX®, Rootella X®, and rhizobium. This was done to optimize plant growth success, to provide a small volume of soil to encourage AM fungi colonization of the roots, and to allow rhizobium to become established prior to transplantation into the H-pots.

2.3.2.3 Block and position randomization

Excel was used to randomize the block allocation for each H-pot with the formula `=unique(randarray(30,,1,10,true))` and to randomly assign H-pots to their position within the blocks using `=index(sortby (#:#,randarray(12(#:#))),sequence(12))`. Every treatment combination appeared once within each block to distribute the effects of confounding factors in the greenhouse microclimates (Gilmour & Trinca, 2012).

2.3.2.4 *Ascochyta rabiei* disease challenge

Chickpeas receiving the challenged treatment were sprayed with 3mL of a conidial spore suspension (~35,000 spores/mL) at flowering. Controls received 3mL of sterile water.

2.3.2.5 Data Collection

Data collection in this experiment consisted of (a) visually rating AB disease in all the plants three weeks after disease challenge, and (b) at the end of the experiment, a random sampling of 18 roots was taken to verify successful AM fungi colonization in both the flax and chickpea roots (Appendix A).

2.3.2.6 Data Analysis

Statistical analyses were completed using R (version 4.1.1, 2021-08-10). Normality for disease ratings was checked using QQ plots and the Shapiro-Wilk test by applying the codes `qqnorm` from base R and `Shapiro.test` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). Homoscedasticity was tested using Levene's test by applying the code `levene_test` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). The Kruskal-Wallis Rank Sum Test was applied to the non-parametric data using the `kruskal.test` function from the `dplyr` package (Kassambara, 2023; R Core Team, 2023). Statistical assumptions for Kruskal-Wallis are (a) the data are non-normal or skewed; (b) the variable being tested has more than two independent groups. Effect sizes were determined with the `epsilonSquared` function (Iacobucci *et al.,* 2023) contained in the `rcompanion` package (Mangiafico, 2023). Data were subsetted by intercrop type (C-C, C-F) and the Kruskal-Wallis test was applied. Significance was assessed at $\alpha=0.05$.

2.3.3 EXPERIMENT B: INVESTIGATING THE CMN WITHIN A DONOR-RECEIVER RELATIONSHIP

2.3.3.1 Experimental design

In a greenhouse located at AAFC in Lethbridge, Alberta, a randomized design was implemented for H-pots consisting of only chickpeas with two hyphal connection manipulation treatments (CMN and BLK), that were all challenged with *A. rabiei* at flowering. One table in the greenhouse was divided into three columns and 10 rows with randomization completed in Excel using the `=rand` function. There were 15 replicates per treatment combination for a total of 30 pots. H-pots were grown with a photoperiod of 12:12 and a temperature of 23ºC daytime, 18ºC night-time, until pathogen challenge when temperature was decreased to a constant 17ºC day and night. The plants were hand-watered by filling their saucers to ensure all plants received the same amount of water.

2.3.3.2 Seed preparation

120 chickpeas were surfaced sterilized in 5.25% hypochlorite for 10 minutes and rinsed four times in sterilized dH_2O. The chickpeas were planted directly into the H-pots filled with PRO-MIX®. Seeds were planted in duplicate to ensure 100% germination with the plant farthest from the connector being culled if both emerged, leaving a single plant in each side of the H-pot. All chickpeas were inoculated with 0.02g rhizobium and 1 mL of Rootella X® as previously described.

2.3.3.3 *Ascochyta rabiei* disease challenge

One chickpea in each pairing was randomly chosen at flowering to receive 3mL of conidial spore suspension (~265,000 spores/mL; “donor”). One week later, the second chickpea in the pairing (“receiver”) received 3mL of conidial spore suspension (~230,000 spores/mL).

2.3.3.4 Data Collection

Data collection in this experiment consisted of (a) visually assessing AB disease severity in the chickpea plants two weeks after challenge, (b) natural plant height, number of flowers, and dry biomass measurements for each plant, and (c) at the end of the experiment, a random sampling of six chickpea roots were analyzed to confirm AM fungi root colonization (Appendix A).

Natural plant height was measured with a ruler by placing the zero flush with the base of the stem at the soil surface, taken at the highest point of the plant on the ruler to the nearest centimetre. The number of flowers before and after disease challenge were counted. After disease ratings were obtained, above-ground plant material for all plants was harvested by cutting above the root at the epicotyl region, placing them in a pre-labelled and pre-weighed paper bag, that were dried in a 60ºC oven for 48 hours. Bags were weighed after drying and biomass was obtained by subtracting the empty bag weight from the final weight.

2.3.3.5 Data Analysis

Statistical analyses were completed using R version 4.3.1 (2023-06-16 ucrt). Normality was checked using the Shapiro-Wilk test by applying the code `Shapiro.test` from the `rstatix` package (R Core Team, 2023). An F-test was used for homogeneity of variance using the function `var.test` (R Core Team, 2023). If data were normally distributed then the two-sample t-test was applied with the code `t.test` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). For non-normal data, the non-parametric Mann-Whitney U test was applied using the `wilcox.test` function (Wickham *et al.,* 2023). Statistical assumptions for the t-test include (a) the data are continuous, (b) the data are

homoscedastic, (c) the data are normal, and (d) the samples are from a random population (JMP Statistical Discovery, 2024). Statistical assumptions for the Mann-Whitney U test include (a) the dependent variable is measured on a continuous scale, (b) the data is non-parametric, (c) samples are all independent, (d) distributions have the same variability, and (e) the sample sizes are small (<30) (IBM Corp., 2023). Non-parametric effect sizes were calculated using Wilcoxon effect size denoted by the symbol 'r' calculated by applying the code `wilcox_effsize` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). Significance was assessed at α=0.05.

2.4 RESULTS

2.4.1 EXPERIMENT A: INVESTIGATING THE CMN WITHIN A CHICKPEA-FLAX INTERCROP

2.4.1.1 Germination rates: chickpea and flax

60% of the chickpeas germinated and 100% of the flax germinated. Throughout the experiment, 33 (26%) chickpeas died prior to disease challenge while all of the flax remained viable.

2.4.1.2 Rhizobium inoculation

There was 100% success with rhizobium inoculation in the chickpea plants evidenced by multiple functional nodules on the roots. Functionality was confirmed by observing the pink coloured nodes that is associated with N fixation.

2.4.1.3 Results of blocking

A Kruskal-Wallis H test was applied to determine if there was a significant effect of blocking on disease ratings. There was no significant difference ($H(9)=9.802$, $P=0.367$, $\varepsilon^2=0.23$; Fig. 6) in disease severity between all the blocks.

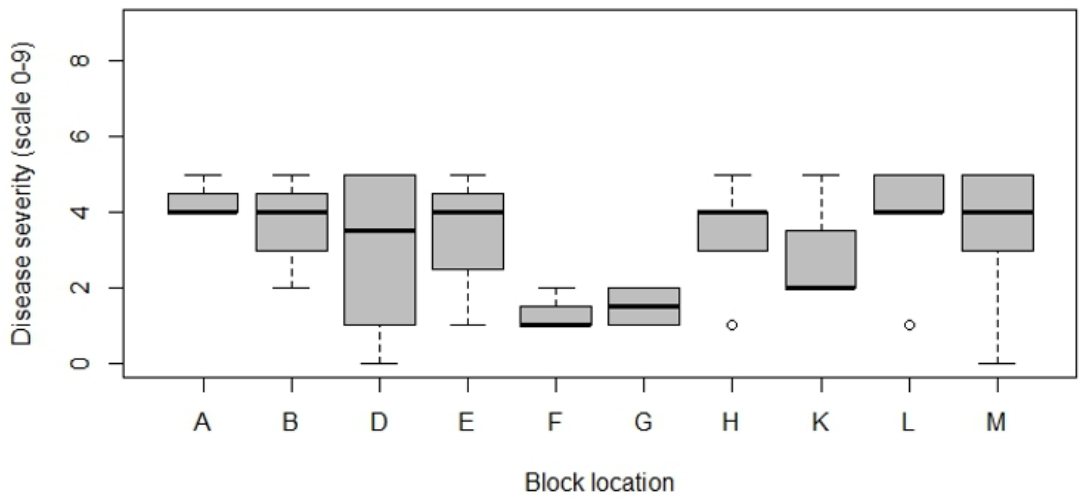


Figure 6. Boxplot for the effect of blocking. There was no significant difference (P=0.367) in disease rating severity between all the blocks.

2.4.1.4 Effect of hyphal connection manipulations on disease severity

A Kruskal-Wallis H test was applied to determine if there was a significant effect of NM, CMN, and BLK treatments on disease severity. The CMN treatment significantly decreased AB disease severity ($H(2)$=7.355, P=0.0253, ε^2=0.175; Fig. 7) with a medium effect size (17%). The BLK and NM treatments had an average disease severity rating of four while the CMN treatment had the lowest with an average disease rating of two. According to field testing, the average disease severity in CDC Leader for AB is 4.6 (Saskatchewan Seed Growers' Association, 2024). It is interesting to note that the NM treatment that permitted root contact as well, did not result in the same decrease in disease severity since it would be assumed a CMN would be created.

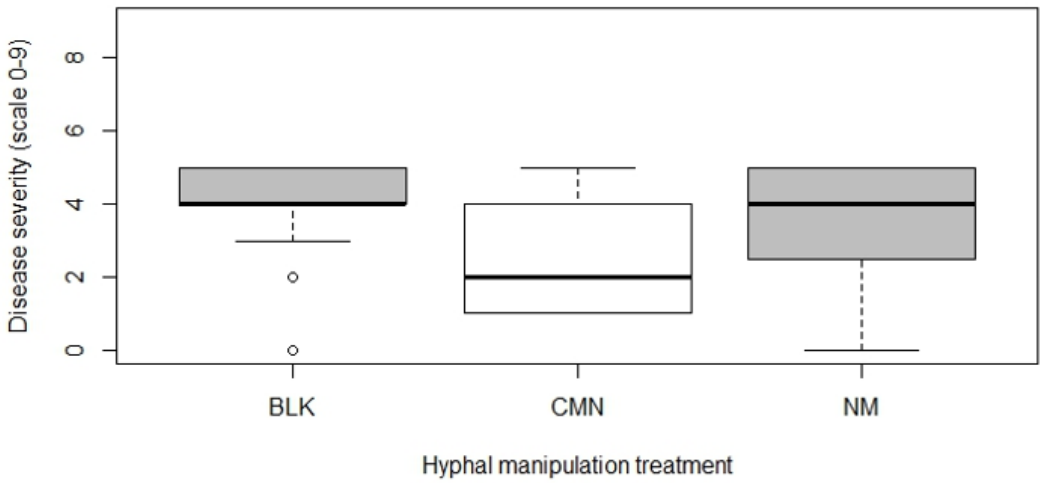


Figure 7. Boxplot for effect of hyphal connection manipulations on *Ascochyta* blight (AB) severity in both chickpea-chickpea and chickpea-flax intercrops. The CMN treatment resulted in a significant decrease (P=0.0253) in disease severity compared to BLK and NM treatments.

2.4.1.5 Effect of intercropping on disease ratings

A Kruskal-Wallis H test was applied to determine if there was a significant effect of intercropping on disease severity. Intercropping chickpea-flax did not significantly affect disease severity ($H(1)$=0.264, P=0.61, ε^2=0.006; Fig. 8). The effect size is almost negligible and the average disease rating for both plant pairings is a four. The range of disease severity is the same, from a zero to a five.

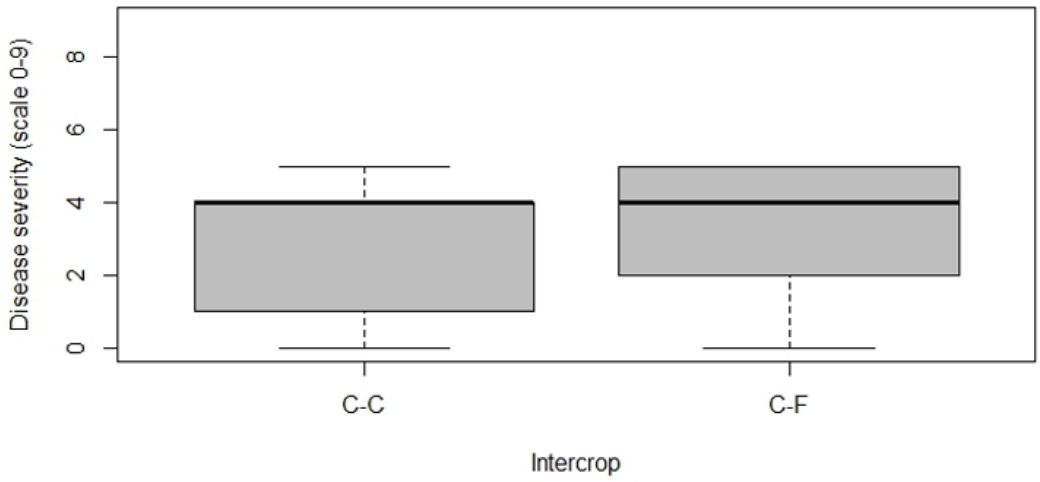


Figure 8. Boxplot for intercropping effect on *Ascochyta* blight (AB) severity. There was no significant difference in disease severity between chickpea-chickpea (C-C) and chickpea-flax (C-F) (P=0.61).

To explore the data further, based on significant results from the CMN treatment, the data were subsetted by intercrop (chickpea-chickpea, chickpea-flax). A Kruskal-Wallis test indicated a significant decrease in the chickpea-flax CMN treatments ($H(2)$=6.49, P=0.039, ε^2=0.31; Fig. 9a) but no significant difference in the chickpea-chickpea CMN treatments (Fig. 9b). Viewing the boxplots (Fig. 9a,b), it is clear the significance is in the CMN treatments, but that the C-C plants actually have a lower disease severity than the C-F. To investigate this, the CMN treatment was subsetted from the original data set (Fig. 9c). A Kruskal-Wallis test was applied that indicated no significant difference between the intercrop plants ($H(1)$=0.24, P=0.62, ε^2=0.017). Therefore, it is the CMN treatment that significantly decreased disease severity.

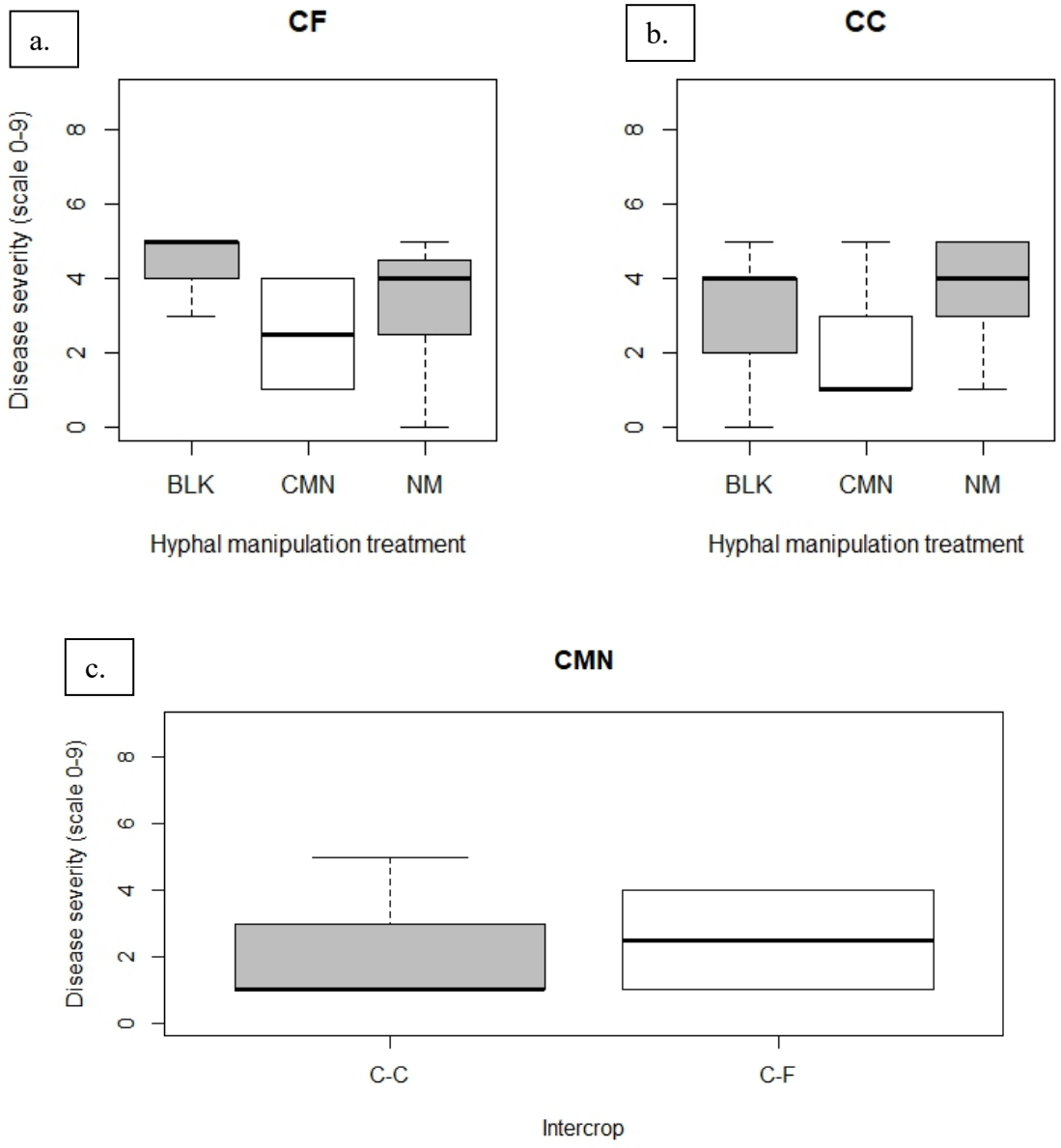


Figure 9. Boxplots for subset data. **(a)** The chickpea-flax (CF) intercrop CMN treatment resulted in a significant decrease (P=0.039) in disease severity compared to BLK and NM treatments, and **(b)** the chickpea-chickpea (CC) pairing resulted in no significant difference between mesh treatments (P=0.21). **(c)** Subset CMN data resulted in no significant difference between the intercrop plants (P=0.62).

2.4.1.6 Aggregate disease ratings

Aggregate disease ratings (Table 1) on the 0-9 scale were determined for the intercrop and hyphal connection manipulation treatments. Chickpea-chickpea intercrop with CMN treatment had the lowest aggregate disease rating. For chickpea-chickpea the highest disease severity was in the treatment that permitted both root contact and hyphal connections. Chickpea-flax intercrop with CMN treatment had the lowest aggregate disease rating. For chickpea-flax the highest disease severity was in the treatment that blocked both root contact and hyphal connections.

Table 1. Aggregate disease ratings for chickpea-chickpea and chickpea-flax intercrops in H-pots based on hyphal connection manipulation (NM, CMN, BLK).

Intercrop: Chickpea-Chickpea	**Hyphal treatment**	**Aggregate disease rating (0-9) ***	**Intercrop:** Chickpea-Flax	**Hyphal treatment**	**Aggregate disease rating (0-9) ***
	CMN	2.14		CMN	2.50
	BLK	3.17		BLK	4.43
	NM	3.75		NM	3.29

*0=plant is alive and not diseased; 9=plant is dead

2.4.1.7 Confirmation of root colonization by AM fungi

Estimation of AM fungi colonization in chickpea and flax roots resulted in an average of 84% across all treatments.

2.4.2 EXPERIMENT B: INVESTIGATING THE CMN IN A DONOR-RECEIVER RELATIONSHIP

2.4.2.1 Chickpea germination rates

The chickpea seed germination rate was 100% in this experiment, representing all 120 seeds. No chickpea plants died throughout the experiment, prior to being challenged with *A. rabiei,* providing 100% survival rate.

2.4.2.2 Rhizobium inoculation

There was 100% success with rhizobium inoculation in the chickpea plants evidenced by multiple functional nodules on the roots. Functionality was confirmed by observing the pink coloured nodes that is associated with N fixation.

2.4.2.3 Effect of hyphal connection manipulation on donor and receiver plant disease severity

A two-sample t-test was performed to compare AB disease severity in the donor plants against hyphal connection manipulations. There was no significant difference ($t(28)=0$, $p=0.50$, $d=0$) in the disease ratings between the donor plants with the CMN treatment and the BLK treatment (Fig. 10a).

A Mann-Whitney U test was performed to compare AB disease severity in the receiver plants against hyphal connection manipulations. There was no significant difference ($W=128.5$, $p=0.49$, $r=0.13$) in the disease ratings between the donor plants with the CMN treatment and the BLK treatment (Fig. 10b).

A two-sample t-test was performed to compare the difference in AB disease severity in the receiver plants against hyphal connection manipulation to their donor plant. A positive (+) difference indicates an increase in disease severity in the receiver plant while a negative (-) difference indicates a decrease in disease severity in the receiver plant. There was no significant difference ($t(28)=0.177$, $p=0.430$, $d=0.065$) in the difference in disease ratings

in the receiver plants (Fig. 10c). The difference in disease severity in the CMN treatment ranged from -5 to +4, whereas the difference in disease severity in the BLK treatment ranged from -2 to +1. The CMN treatment saw the biggest decrease in receiver plant disease severity.

2.4.2.4 Effect of hyphal connection manipulation on plant height

A Mann-Whitney U test was performed to compare donor plant height against hyphal connection manipulations. There was no significant difference ($W=141.5$, $p=0.23$, $r=0.22$) in height between the donor plants with either the CMN or the BLK treatment (Fig. 11a).

A Mann-Whitney U test was performed to compare receiver plant height against hyphal connection manipulations. There was no significant difference ($W=128.5$, $p=0.49$, $r=0.13$) in height between the receiver plants with either the CMN or the BLK treatment (Fig. 11b).

A Mann-Whitney U test was performed to compare differences in chickpea receiver plant height to their donor based on their hyphal connection treatment. Positive numbers indicate an increase in plant height and negative numbers indicate a decrease in plant height. There was no significant difference ($W=120$, $p=0.77$, $r=0.057$) in plant height in either the receiver plants that received the CMN treatment or the BLK treatment (Fig. 11c).

The heights of the receiver plants in the CMN treatment had a median of 30 cm with a range of 21 – 33 cm compared to their donors that had a median of 27 cm with a range of 25.5 – 32 cm. The heights of the receiver and donor plants in the BLK treatment had the same median of 30 cm with a range of 25 – 34 cm and 25.5 – 33 cm respectively.

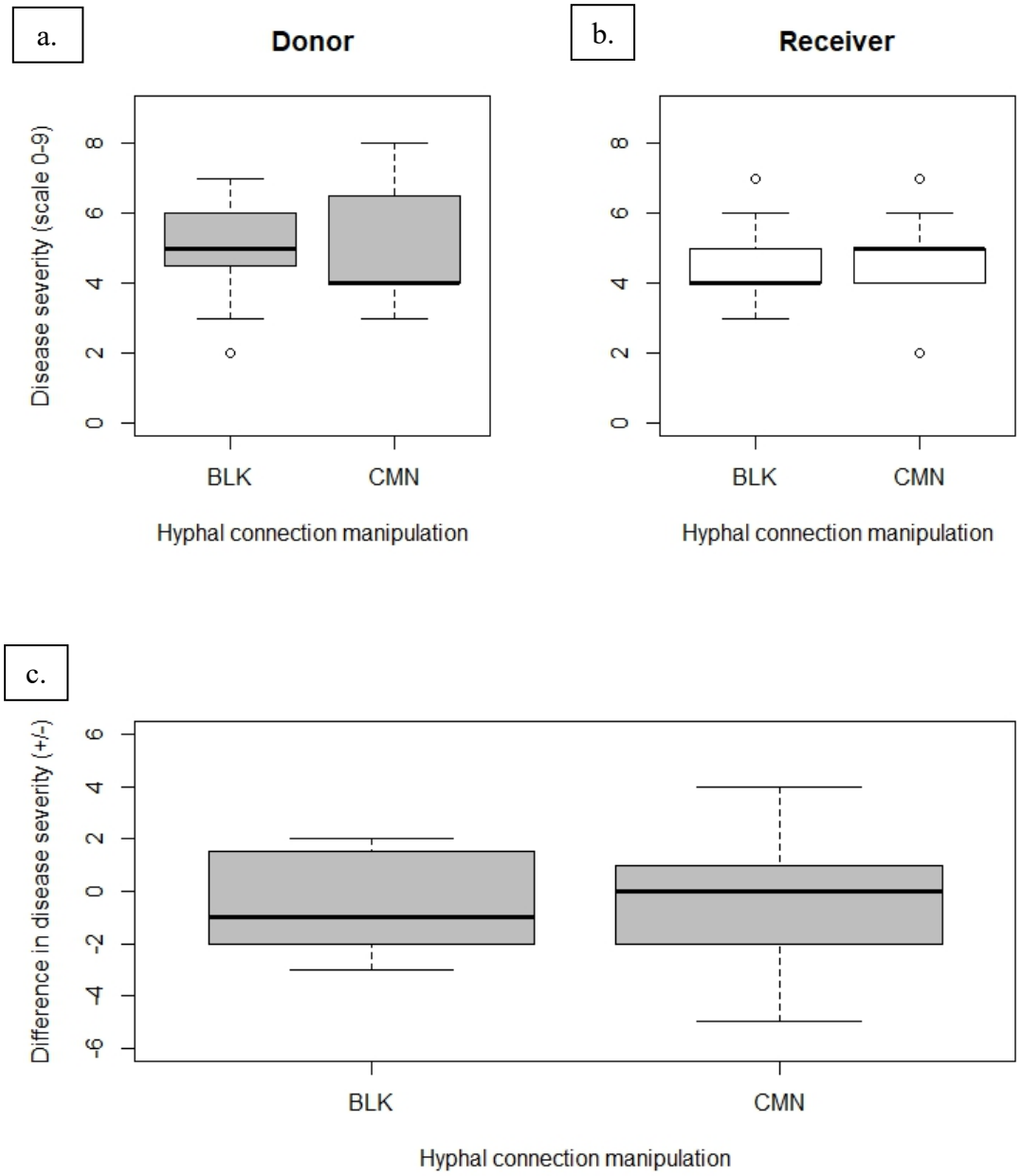


Figure 10. Boxplots for *Ascochyta* blight (AB) severity in donor and receiver chickpea plants. Plants received either CMN (n=15) or BLK (n=15) treatments. There was no significant difference in disease severity in either **(a)** the donor (p=0.430) or **(b)** receiver plants (p=0.49). **(c)** Difference in AB severity in the receiver chickpea plants compared to their donor plants. Positive number indicates an increase in disease severity and a negative number indicates a decrease in disease severity. There was no significant effect of the donor on disease severity in the receiver plants (p=0.430).

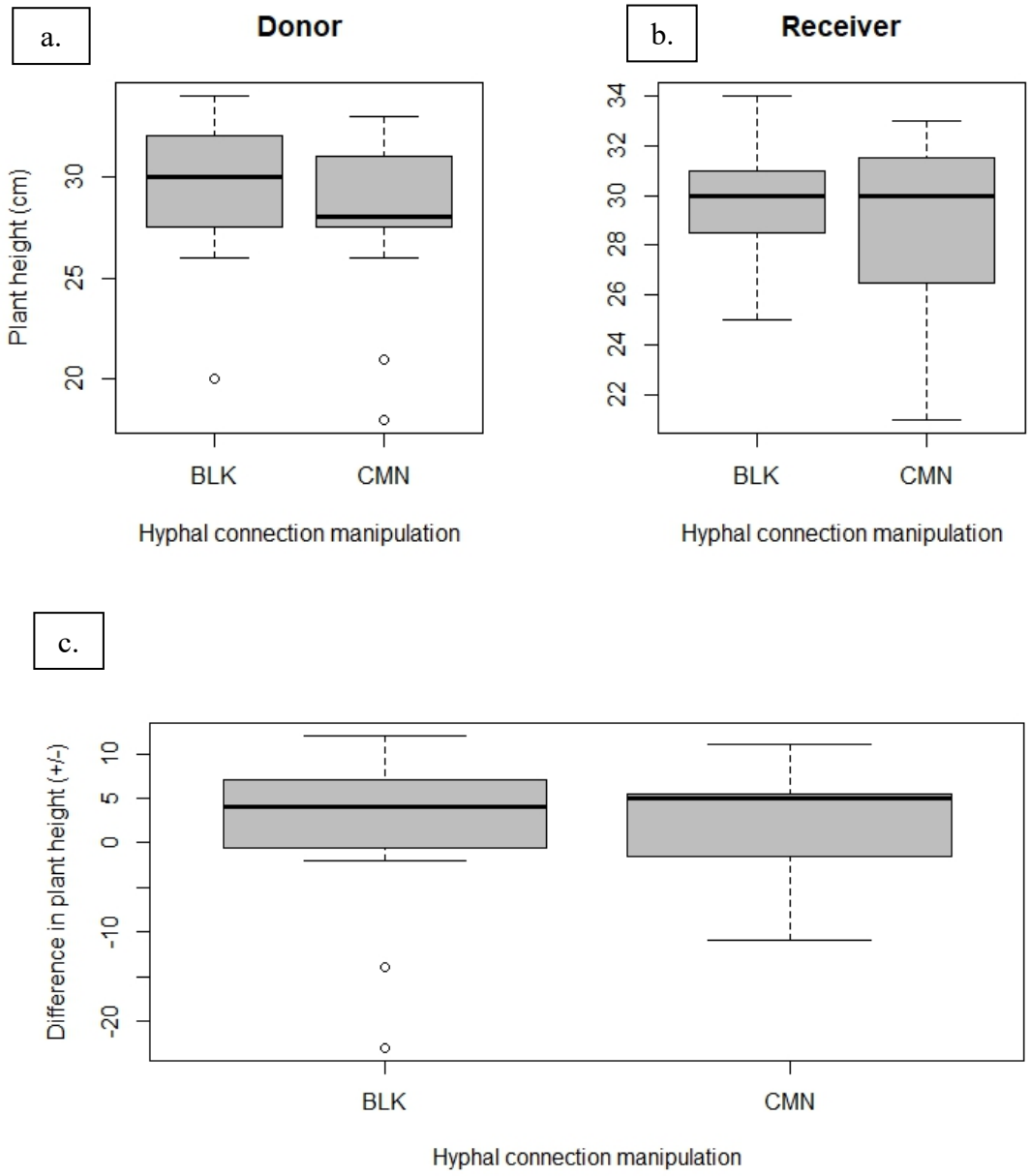


Figure 11. Boxplots for plant height in donor and receiver chickpea plants. Plants received either CMN (n=15) or BLK (n=15) treatments. There was no significant difference in plant height in either **(a)** the donor (p=0.50) or **(b)** receiver plants (p=0.49). **(c)** Difference in plant height in the receiver chickpea plants compared to their donor plants. Positive number indicates an increase in height and a negative number indicates a decrease in height. There was no significant effect of the donor on height in the receiver plants (p=0.77).

2.4.2.5 Effect of hyphal connection manipulation on plant above-ground dry biomass

A two-sample t-test was performed to compare chickpea above-ground dry biomass in the donor plants against hyphal connection treatment. There was no significant difference ($t(28)=-0.798$, $p=0.784$, $d=-0.29$) in the disease ratings between the donor plants that received CMN or BLK treatments (Fig. 12a).

A Mann-Whitney U test was performed to compare chickpea above-ground dry biomass the receiver plants against hyphal connection treatment. There was no significant difference ($W=108$, $p=0.870$, $r=0.01$) in the disease ratings between the receiver plants that received CMN or BLK treatments (Fig. 12b).

A Mann-Whitney U test was performed to compare differences in chickpea receiver plant biomass compared to their donor based on their hyphal connection treatment. Positive numbers indicate an increase in plant biomass and negative numbers indicate a decrease in plant biomass. There was no significant difference ($W=106.5$, $p=0.82$, $r=0.045$) in plant biomass in the receiver plants that received the CMN or the BLK treatments (Fig. 12c).

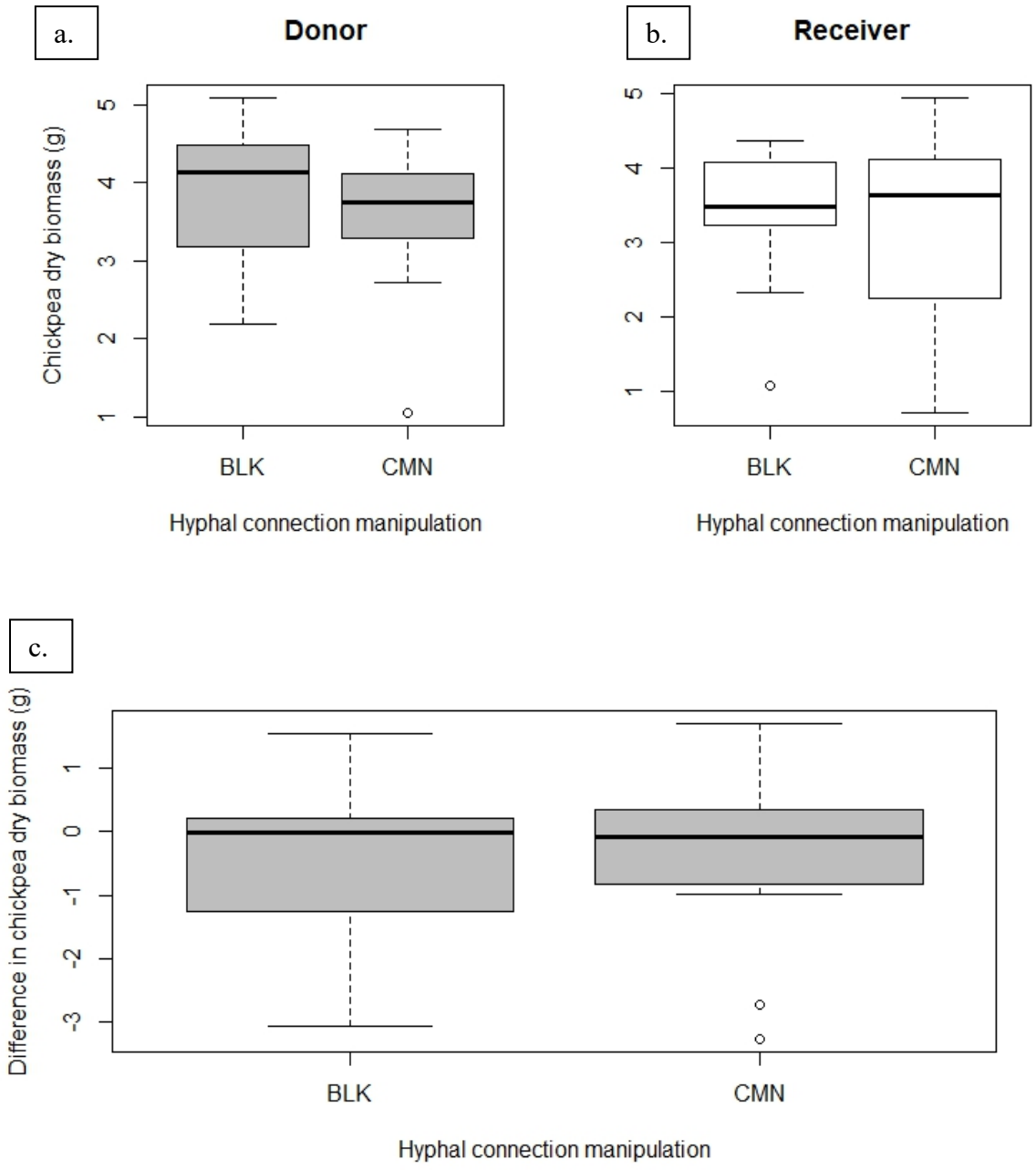
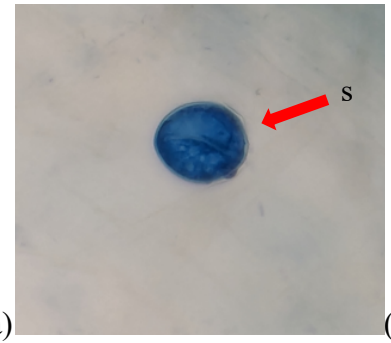


Figure 12. Boxplots for biomass in donor and receiver chickpea plants. Plants received either CMN (n=15) or BLK (n=15) treatments. There was no significant difference in biomass in either **(a)** the donor (p=0.784) or **(b)** receiver plants (p=0.870). **(c)** Difference in biomass in the receiver chickpea plants compared to their donor plants. Positive number indicates an increase in biomass and a negative number indicates a decrease in biomass. There was no significant effect of the donor on biomass in the receiver plants (p=0.82).

2.4.2.6 Confirmation of root colonization by AM fungi

Estimation of AM fungi colonization in chickpea roots resulted in an average of 85% between both mesh treatments. Individually, the CMN treatment resulted in 92% colonization and the BLK treatment resulted in 78% colonization (Appendix A). Most structures identified were spores with a thick wall ('s', Fig. 13a) and vesicles ('v', Fig. 13b).

(a) 

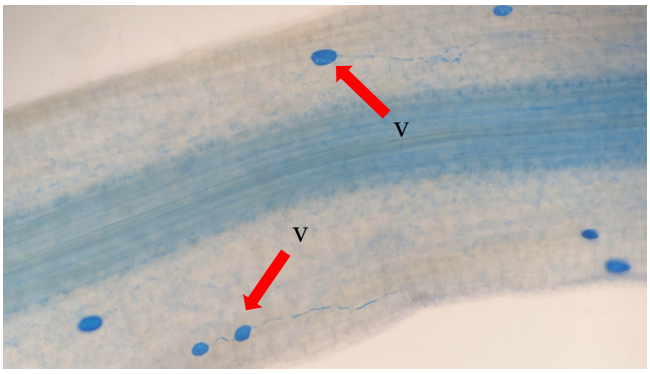
(b) 

Figure 13. AM fungi structures dyed with Trypan blue identified using a light microscope at 40x magnification. **(a)** 's' for spores, and **(b)** 'v' for vesicles. Obtained from root sample C6 (AMF inoculated).

2.5 DISCUSSION

These experiments were conducted to investigate whether the flax and/or the CMN plays a role in intercropped chickpea and flax (Experiment A) and whether the CMN plays a role in a chickpea-chickpea donor-receiver relationship (Experiment B) when the donor is challenged one week prior to the receiver. It was found that although the presence of flax was not a significant effect, the CMN appears to play a role in intercropping, and the CMN does not play a role in a donor-receiver relationship specifically at the one-week timepoint.

In Experiment A, there was a significant decrease in disease severity (Fig. 7) in the CMN treatment in both the chickpea-chickpea and chickpea-flax pairings, but not in the NM

treatment. This result might be explained by way of root competition. There is evidence that in the presence of AM fungi, roots from different plant species will suppress, through allelic root exudates, symbiotic interactions (Achatz & Rillig, 2014; Barto *et al.,* 2011). Plants in the H-pots experienced root binding with high heat due to the spring sun shining into the greenhouse, high humidity for disease development, nutrient limitation due to the small pot size, and at times water stress combined with pathogen attack. Water stress would occur for plants that were more in the sun than other plants, but all plants were watered at the same time. AM fungi potentially supported plant health in the presence of these stressors.

There was no significant difference between the blocks on the greenhouse tables (Fig. 6). Blocks F and G did have the lowest disease severity most likely due to them being in the middle of the greenhouse, and in May/June the sun was shining most strongly on them, drying out the soil faster than the rest of the blocks, so the plants were very dry at rating. The plants were all watered the same amount on the same days and was not adjusted for sun exposure. *Ascochyta rabiei* is a necrotrophic pathogen that kills living plant tissue to extract nutrients from it; therefore, if the plants were already dry or dead due to sun-heat induced drought, the pathogen would not be able to properly infect the plant and disease ratings would be artificially lower. Statistical analyses were conducted with and without block F, however, there was no significant difference between the two results, so block F was left in the analysis as only one replicate from each treatment group was affected.

Mycorrhizal spores germinate anywhere from 24 hours to 14 days depending on the species (Maia *et al.,* 1994). Fungal hyphae have been studied and found to extend between 0.4-17 mm day^{-1} (Watt *et al.,* 2006) while the most common species of AM fungi, *Claroideoglomus etunicatum*, was found to extend at 4.1 mm day^{-1} (Schütz *et al.,* 2022).

The H-pot connector was an average of four inches long. With the roots confined to their compartments with the mesh, it would have taken approximately 13-20 days for the CMN to be established if we consider that each roots hyphae will extend half the distance and meet in the middle. The plants were challenged when the chickpeas flowered as this is when genetic resistance declines (Singh & Reddy, 1993). Flowering occurred at 41 days for the intercropping experiment and at 65 days for the donor-receiver experiment (53 days in the field (Saskatchewan Seed Growers' Association, 2024)). These are both more than sufficient time for a CMN to have developed.

Based on previous intercropping experiments in the fields of Saskatchewan and the USA where chickpea-flax crops experienced a reduced disease severity, it was unexpected that there was no difference in disease severity between the plant pairings of chickpea-chickpea and chickpea-flax (Fig. 7). A possible explanation for this is that in a field environment with complexities of the soil, there are many more factors interacting between the rhizospheres, soil microbiome, and the environment itself (sunlight, nutrients, moisture, wind etc). Since there was no significant effect of plant pairing, perhaps there is another mechanism acting as a suppressor of disease when these plants are intercropped that is not present in the greenhouse. It is feasible that the taller flax provides a mechanical barrier to disease progression within the fields when intercropped with chickpea rather than a soil or root-mediated resistance mechanism. In the greenhouse, the purpose of planting one flax with one chickpea was to isolate any effects of the flax on suppressing AB disease in the chickpeas in the rhizosphere via the CMN.

One sample, donor 1005 with a disease rating of three, produced new shoots, leaves, and a bud, suggesting that at low disease severity, AM fungi may further support plant recovery during disease. Many studies have investigated the ability of AM fungi to support plants

during stress (Bahadur *et al.,* 2019; Begum *et al.,* 2019; Chandrasekaran & Paramasivan, 2022; Dowarah *et al.,* 2022).

Experiments with the CMN have shown that potential defense signals are transferred between plants, specifically in a donor and receiver relationship, that may inform the receiver of impending attack (Song *et al.,* 2010, 2013). Defense enzymes were upregulated in a receiver plant when connected to a CMN 65 hours after the donor plant was challenged with a pathogen, suggesting disease resistance can be transferred through the CMN (Song *et al.,* 2010). Defenses were also upregulated in tomato plants against herbivore pests in plants connected via a CMN (Gilbert, *et al.,* 2013). This was explained by way of interplant signalling that could have only occurred through the CMN acting as a conduit for transmission of signals (Johnson & Gilbert, 2015). Although previous research has shown that the CMN has the ability to transfer signals (be it chemical, electrical, or other) (Gilbert, *et al.,* 2013; Babikova, Johnson, *et al.,* 2013, 2014; Song *et al.,* 2010, 2014; Volkov & Shtessel, 2020), this study did not find evidence that this occurs in a chickpea-chickpea H-pot greenhouse system when receiver plants are challenged one week after a donor plant.

In Experiment B, there was no significant difference in disease severity between the donor plant or receiver plant when connected to the CMN (Figs. 10a,b,c). There was also no significant difference in plant height (Figs. 11a,b,c) or biomass (Figs. 12a,b,c) in either the donor or receiver chickpea plants when either connected to a CMN or not. However, AM fungi have not been found to affect biomass when an abundance of soil nutrients exist (Hoeksema *et al.,* 2010).

It is interesting to note that the AM fungi colonization within the chickpea roots connected via a CMN was 92% versus the BLK treatment (78%). This could be interpreted as action of the CMN since previous research found that when one side of a microcosm

was inoculated with AM fungi and the other was uninoculated, the only way AM fungi was detected in the uninoculated compartment was due to the CMN connection (Li *et al.,* 2022).

The difference in donor-receiver plant heights, with the receiver in the CMN treatment having a higher median height (30 cm) than the donor (27 cm) may have resulted from the CMN transferring nutrients from the donor plants to the receiver, or defense signals being received that reinforced photosynthetic structures, allowing them to grow taller. Even though this result is not significant, it is still an interesting finding. In the BLK treatment, the median heights for both donor and receiver were the same (30 cm), further supporting the possibility that the CMN was transferring something of benefit to the receiver from the donor. CDC Leader chickpeas when grown in the field reach heights of 41 cm (Saskatchewan Seed Growers' Association, 2024). The small volume of the H-pot most likely contributed to a decrease in plant height by an average of 11 cm in this experiment.

Differing levels of pathogen pressure were used in Experiment A and Experiment B since in the field, disease pressure will vary, and plant responses follow. The typical airborne spore load per day during a disease outbreak year can be as high as 100 spores with a yearly total of >400,000 (Grinn-Gofroń *et al.,* 2020). As temperatures continue to climb and rain splash combine to release fungal spores into the environment, the expectation is that potentially higher pathogenic fungal spore loads will increase daily (Grinn-Gofroń *et al.,* 2020), causing more incidences of *Ascochyta rabiei* in chickpea crops worldwide.

2.5.1 Constraints

In these experiments it was necessary to remove the complexities of soil by growing the chickpea and flax plants in a potting mix. This also meant that the natural AM fungi community present in soil needed to be artificially inoculated with a commercial inoculum

into the potting mix. Although the potting mix contained one propagule per gram of mix this is not sufficient for these experiments. In addition, disease ratings are based on the observer's subjective opinion, and therefore, if another observer were to make these ratings, the data could potentially be different. There is also no method currently available to confirm creation of a CMN other than measuring differences in plant traits and disease severity between CMN and BLK treatments. There could have been secretions and exudates in the rhizosphere and bulk soil that were transported across the mesh within water molecules ($2.75x10^{-4}$ µm) that could not be prevented due to the nature of the experiment. Some root exudates are small enough (Zhang *et al.,* 2022) to pass through the blocking mesh.

2.5.2 Future Research

Future research can focus on conducting a chickpea-flax intercrop with only BLK and CMN treatments to determine if when disease pressure is applied, the action of the CMN is still a significant effect. In addition, matching AM fungi species and their ability to form CMNs between different intercrop pairings specific to each crop disease (e.g., bacterial, fungal, nematode, viral) may provide interesting results. Plant-pathogen communication methods, whether via the CMN or exudates, are a crucial area of research for sustainable crop protection methods since an understanding of these can lead to novel disease management solutions.

Conducting field experiments with natural populations of AM fungi in soil including size exclusion mesh for intercropped and monocropped chickpeas would provide more concrete evidence of the effectiveness of AM fungi in reducing AB disease severity of chickpeas based on these greenhouse results, to further evaluate AM fungi effectiveness as a disease reduction mechanism.

The initial premise for this study was the question of why mixed row intercropping of chickpea and flax most often result in a decrease in AB severity. Therefore, future research should include flax-chickpea pairs where flax is challenged first and then chickpea to determine if there are any defense chemicals synthesized by the non-host flax that are being received or perceived by chickpea, priming its defense mechanisms.

2.5.3 Conclusion

In the face of a changing climate, pathogen evolution, and pesticide insensitivity, novel methods of AB disease control are required. Chickpea crops are highly susceptible to AB as genetic resistance is low and lowers further at flowering. In this study, AM fungi appear to have a role through the CMN in reducing AB disease pressure in the greenhouse. Future studies should focus on whether this mechanism remains active in the field and whether different species of AM fungi confer the same benefit to different varieties of chickpea that could form part of an integrated pest management system.

CHAPTER 3: CHICKPEA (CDC LEADER) INOCULATED WITH ARBUSCULAR MYCORRHIZAL FUNGI (AM FUNGI) DECREASED ASCOCHYTA BLIGHT DISEASE SEVERITY

3.1 ABSTRACT

Chickpeas (*Cicer arietinum*) are susceptible to a devastating pathogenic foliar fungus, *Ascochyta rabiei*, the causal agent of *Ascochyta* blight (AB). In outbreak years, this pathogen can cause up to 100% crop loss. Arbuscular mycorrhizal fungi (AM fungi), a plant symbiont, supports plants encountering biotic and abiotic forms of stress. Arbuscular mycorrhizal fungi provision nitrogen and phosphorus, stimulate the production of plant secondary metabolites, and support activation of defense signaling. As such, AM fungi have the potential to be a sustainable inoculant in the integrated pest management of AB. **Purpose:** To test the hypothesis that chickpea plants inoculated with AM fungi would have a significantly lower AB disease severity, increased height, number of flowers, and biomass than chickpea plants that were not inoculated. **Methods:** A greenhouse experiment was conducted. Chickpea seeds were planted in single pots in a soilless medium and either inoculated with AM fungi (n=9) or not (n=9) (*Rhizophagus irregularis; Funneliformis mosseae*). *Ascochyta* blight was induced by spraying an *A. rabiei* conidial spore suspension onto the plants at flowering. Plant height before and after disease challenge was recorded, along with number of flowers, above-ground dry biomass, and visual disease ratings two weeks post-challenge. **Results:** There was a significant decrease in disease severity in the plants inoculated with AM fungi but no significant difference in number of flowers, biomass, or height between the treatments. Therefore, it is likely that AM fungi support chickpea plants before and during AB disease.

3.2 INTRODUCTION

Chickpeas (*Cicer arietinum*) are an economically important crop that is susceptible to *Ascochyta* blight, a devastating foliar fungal disease caused by *Ascochyta rabiei* (teleomorph *Didymella rabiei*) (Foresto *et al.,* 2023; Harveson *et al.,* 2011). *Ascochyta* blight is currently managed with foliar fungicides, the most commonly used being from the strobilurin class, derived from basidiomycetes fungi (Bartlett *et al.,* 2002). The strobilurins work with a specific mechanism of action against the cytochrome bc_1 complex called quinone outside inhibitors (QoI) that prevent adenosine triphosphate production (ATP) in the fungus (Bartlett *et al.,* 2002). The QoI fungicides have been used on chickpea crops for blight control since 2002; however, in 2004 insensitivity was already an issue in Canada (Ahmed *et al.,* 2007; Chang *et al.,* 2007; Owati *et al.,* 2017). This insensitivity is due to a mutation in the cytochrome b gene (G143A) (Delgado *et al.,* 2013). In addition, chickpea genetic resistance is insufficient and not durable, and is easily overcome by *A. rabiei* (Ilyas *et al.,* 2022; Reddy & Singh, 1984).

Arbuscular mycorrhizal fungi (AM fungi) are a plant symbiotic soil-borne fungus that exchanges nutrients within roots for plant photosynthetic carbon (Kuyper & Jansa, 2023; Parniske, 2008). Arbuscular mycorrhizal fungi have been shown to support plant defenses (Fontana *et al.,* 2009; Jung *et al.,* 2012; Song *et al.,* 2013; Yu *et al.,* 2022) through mechanisms including regulation of secondary metabolite production, alteration of root morphology, outcompeting pathogens for nutrients, and inducing systemic defenses (Pozo *et al.,* 2002; Weng *et al.,* 2022a). Phytochemicals are also produced on the surface of AM fungal hyphae that increase plant phenol production to support the activation of systemic resistance by promoting the synthesis of important defense phytohormones including ethylene (ET), salicylic acid (SA), and jasmonic acid (JA) (Jung *et al.,* 2012; Weng *et al.,*

2022a). Arbuscular mycorrhizal fungi have a balancing effect on these hormones by affecting gene expression within important signal transduction pathways (Weng *et al.,* 2022a). Outside of the plant, AM fungi attract soil microbes that benefit plant growth while decreasing disease pressure (Cameron *et al.,* 2013). Phosphate solubilizing bacteria (PSB) are an AM fungi endosymbiont that liberate bound P in the soil matrix and transport P in the hyphosphere to plant roots along the growing AM fungal hyphae (Faghihinia *et al.,* 2022; Wang *et al.,* 2016). 'Damage compensation' has also been observed where the plant's ability to recover after damages by pathogen or insect pest is strengthened by the symbiosis (Pozo & Azcon-Aguilar, 2007).

Chickpeas possess natural resistance against AB that relies on putative resistance genes found to be controlled by the plant's circadian clock including susceptibility to pathogens and activation of gene transcripts (Andam *et al.,* 2020; Sharma & Bhatt, 2015). Circadian gating – controlling the circadian cycle – allows the plant to utilize genes most efficiently; for example, defense genes are activated when pathogen attack is at its highest level rather than being constantly activated (Sharma & Bhatt, 2015). Innate responses to other pathogenic fungi include production of hydrolytic enzymes, antimicrobial phytoalexins, and antioxidants to protect against the plant induced reactive oxygen species (ROS) that act as secondary messengers for downstream defense activation (Thakur *et al.,* 2023).

This greenhouse experiment investigated whether inoculation with AM fungi would support chickpea plant growth and reduce disease severity against the fungal pathogen *A. rabiei*. My hypotheses were that (a) AB disease severity would be significantly lower in plants inoculated with AM fungi, and (b) plant morphological characteristics (number of flowers, natural height, and above-ground dry biomass) would be significantly higher in

plants inoculated with AM fungi. These results are important preliminary findings for future research in sustainable chickpea production and AB disease management.

3.3 METHODS

3.3.1 Experimental design

A randomized greenhouse experiment was conducted between October 2023 and January 2024 located at AAFC in Lethbridge, Alberta. Nine chickpea plants were inoculated with AM fungi and nine plants were controls that were not inoculated for a total of 18 pots. All chickpea seeds were planted in a soilless potting medium provided by the AAFC greenhouse (recipe Appendix B). Pots were randomized in Excel using `=rand()`. The photoperiod was 12:12 and the temperature from planting to chickpea flowering was 23ºC daytime, 18ºC night-time, and during pathogen challenge was decreased to a constant 17ºC. Randomized pot placement is shown in Figure 14.

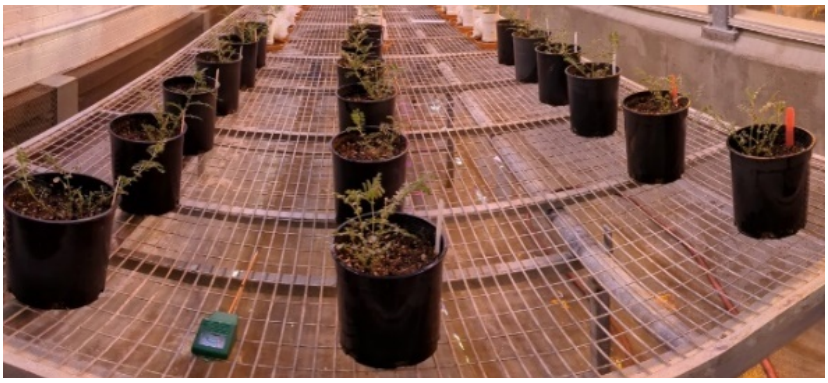


Figure 14. Randomized table positions for pot placement in the greenhouse. Plants were either inoculated with AM fungi (n=9) or not (n=9).

3.3.2 Organisms

This experiment was performed using CDC Leader Kabuli chickpea variety, *Ascochyta rabiei* strain AR-2019-008, commercial AM fungi inoculant (Rootella® X, Groundwork BioAg, Israel) consisting of 133,600 propagules per gram of *Rhizophagus intraradices* and 33,400 propagules per gram of *Funneliformis mosseae* spores, rhizobium species *Mesorhizobium ciceri* (AgroPlus, Coaldale, AB), and AAFC soilless potting mix.

3.3.3 Inoculation of soil with AM fungi and rhizobium

Rootella® X was prepared according to the manufacturer's directions: a working solution of 0.5 g xanthan gum was mixed in 1 L of sterilized water, 100 mL of which was used to mix with 1 g of Rootella® X. 1 mL of the inoculant solution, that contained 500 spores, was applied using a 1mL pipette approximately 2.54 cm in the soil of the treatment group, above which the seed was planted. Rhizobium inoculant (0.02 g) was applied to both treatment and control chickpea seeds to support root nodule formation for nitrogen fixation (Tavasolee *et al.,* 2011).

3.3.4 *Ascochyta rabiei* growth and conidial spore suspension preparation and application

Ascochyta rabiei strain AR-2019-008 was obtained from AAFC, Swift Current in October 2022. This fungus was grown on potato dextrose agar (PDA) plates without antibiotics and stored in the dark at 22°C to encourage spore formation to be used as inoculum. The spore suspensions were made fresh on each morning before plant challenge. Autoclaved distilled water was poured over the *A. rabiei* fungal mat in the petri dish. A hockey stick spreader was used to dislodge the conidial spores from the mat into the water that was collected in a sterile bottle. This process was repeated until 100 mL of spore suspension was captured. A haemocytometer allowed for estimation of spore numbers in

solution under a light microscope, adjusting the concentration using sterile water until the spore suspension was ~6.5x10^4 spores/mL (Kaiser, 1973). This was poured into a sterile spray bottle that released 1mL per spray.

3 mL of *A. rabiei* spore suspension was sprayed on both the inoculated and uninoculated chickpea plants at flowering in a 'dirty' lab to avoid contaminating the greenhouse. To provide a high-humidity environment for disease establishment, all plants were covered with their own transparent Sun bag (B7026, MilliporeSigma, Canada) that had a Poros stainless steel filter for gas exchange (0.02 µm pore size). *Ascochyta rabiei* spores are approximately 3 µm in size (Onosato *et al.,* 2022) so they cannot exit through the Sun bag.

3.3.5 Disease ratings

As described on page 24.

3.3.6 Plant morphological characteristic measurements

As described on page 30.

3.3.7 Data analysis

Statistical analyses were completed using R version 4.3.1 (2023-06-16 ucrt). Normality was checked using the Shapiro-Wilk test by applying the code `Shapiro.test` from the `rstatix` package (Kassambara, 2023) (R Core Team, 2023). If data were normally distributed then the two-sample t-test was applied with the code `t.test` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). If data were non-parametric the Mann-Whitney U test was applied to the data with the code `wilcox.test` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). Homoscedasticity was tested using Levene's test by applying the code `levene_test` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). Statistical assumptions for the t-test include (a) the data are

continuous, (b) the data are homoscedastic, (c) the data are normal, and (d) the samples are from a random population (JMP Statistical Discovery, 2024). Statistical assumptions for the Mann-Whitney U test include (a) the dependent variable is measured on a continuous scale, (b) the data is non-parametric, (c) samples are all independent, (d) distributions have the same variability, and (e) the sample sizes are small (<30) (IBM Corp., 2023). Parametric effect sizes were calculated using Cohen's d denoted by the symbol 'd' calculated by applying the code `%>% cohens_d` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). Non-parametric effect sizes were calculated using Wilcoxon effect size denoted by the symbol 'r' calculated by applying the code `wilcox_effsize` from the `rstatix` package (Kassambara, 2023; R Core Team, 2023). Significance was assessed when α=0.05.

3.4 RESULTS

3.4.1 Rhizobium inoculation

There was no evidence of successful rhizobium inoculation in this experiment due to a lack of visible nodules on the chickpea roots in all 18 plants.

3.4.2 Effect of AM fungi inoculation on disease severity

Inoculation was so successful that pycnidia were observed growing on the pods of the chickpea plants in the diagnostic bullseye pattern (Fig. 15). To determine if AM fungi had any significant effect on disease severity in chickpea plants challenged with *A. rabiei*, the non-parametric Mann-Whitney U test was conducted. The results indicated that there was a significant decrease in disease severity (W=17, p=0.0351, r=0.51; Fig. 16) between chickpea plants inoculated with AM fungi ($\overline{x}$=7) and those uninoculated ($\overline{x}$=8).

Figure 15. Pycnidia (black dots) growing on inoculated chickpea pod in sample C6. Pycnidia house infective conidial spores. The diagnostic bullseye pattern is evident (red box).

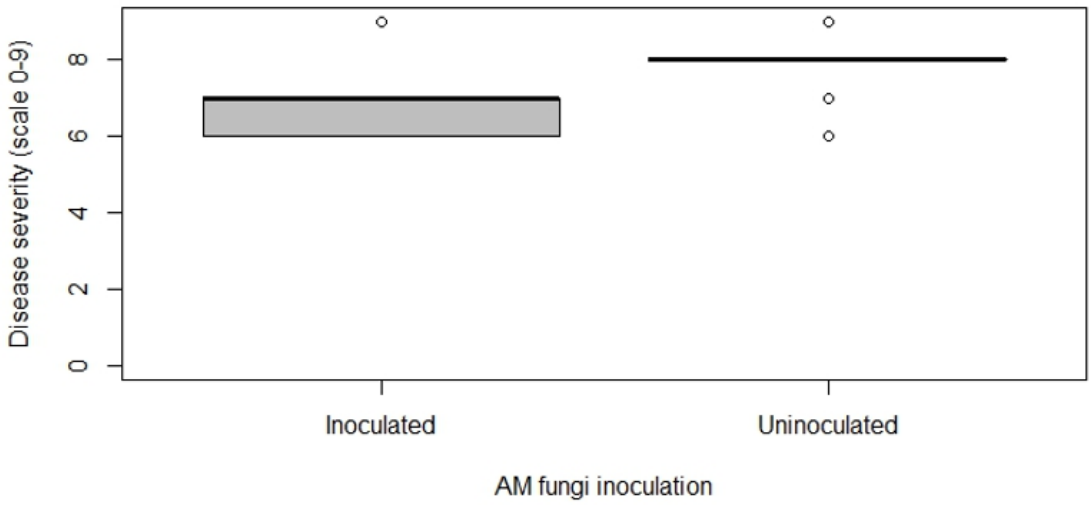


Figure 16. Boxplot for *Ascochyta* blight (AB) disease severity on a 0-9 scale (0: no disease; 9: plant dead) and arbuscular mycorrhizal (AM fungi) plant inoculation status. There is a significant decrease in AB disease in plants that were inoculated with AM fungi (p=0.0351).

3.4.3 Effect of AM fungi inoculation on plant height

A Shapiro-Wilk test revealed a normal distribution for chickpea plant height before disease challenge (p=0.21) and after disease challenge (p=0.29), and for the difference in

plant height (p=0.35). Levene's test revealed the data to be homoscedastic for chickpea plant height before disease challenge (p=0.26) and for the difference in plant height (p=0.55) but was heteroscedastic for plant height after disease challenge (p=0.01). The data were subjected to transformations; however, none could resolve the heteroscedasticity completely.

A two-sample t-test was conducted on chickpea plant height before disease challenge to determine if there was any effect of AM fungi inoculation. The results indicated that there was no significant difference (t(16)=1.57, p=0.068, d=0.74; Fig. 17a) in chickpea plant height before disease challenge in inoculated plants ($\overline{x}$=23.56 cm) versus uninoculated plants ($\overline{x}$=21.34 cm). A Welch's t-test was conducted on chickpea plant height after disease challenge to determine if there was an effect of AM fungi inoculation. The results indicated that there was no significant difference (t(9.14)=0.31, p=0.383, d=0.15, Fig. 17b) between inoculated plants ($\overline{x}$=25.45 cm) and uninoculated plants ($\overline{x}$=25.00 cm). A two-sample t-test was conducted on the difference in height before and after disease, to determine if there was any effect of AM fungi inoculation. The results indicated that there was no significant difference (t(16)=-1.50, p=0.924, d=-0.708, Fig. 17c) in the difference between heights of chickpea plants before and after disease challenge between inoculated plants ($\overline{x}$=1.89 cm) and uninoculated plants ($\overline{x}$=3.67 cm).

3.4.4 Effect of AM fungi inoculation on number of flowers

A Shapiro-Wilk test revealed a normal distribution for number of chickpea plant flowers before disease challenge (p=0.33) and a non-normal distribution after disease challenge (p=0.000). Levene's test revealed the data to be heteroscedastic before disease challenge (p=0.075) and homoscedastic after disease challenge (p=0.21). A natural log

transformation was applied to the number of flowers before disease challenge data, after which p=0.13.

A two-sample t-test was conducted to determine if there was any effect of AM fungi inoculation on the number of flowers before disease challenge. The results indicated that there was no significant difference ($t(16)=0.30$, $p=0.385$, $d=0.14$; Fig. 18a) between plants inoculated ($\overline{x}=6$) with AM fungi and those uninoculated ($\overline{x}=7$). After disease, there were only two plants that still had flowers in the inoculated treatment: sample C6 had three flowers while sample C2 had one flower. The non-parametric Mann-Whitney U test was conducted on the number of flowers after disease. The results indicated that there was no significant difference ($W=49.5$, $p=0.17$, $r=0.34$; Fig. 18b) between plants that were inoculated ($\overline{x}=0.45$) and those that were uninoculated ($\overline{x}=0.00$).

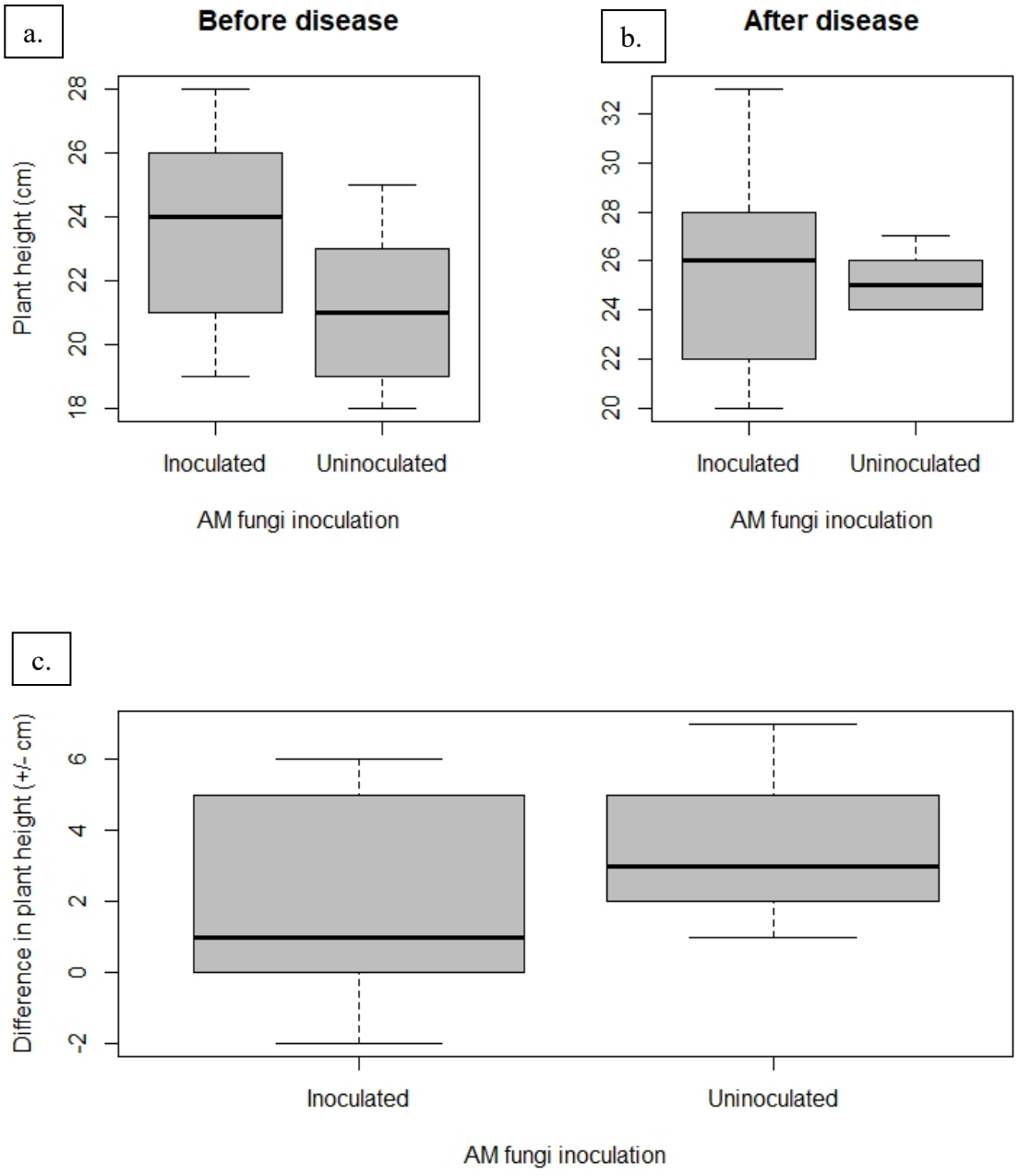


Figure 17. Boxplot for chickpea plant height based on AM fungi inoculation (n=9) or no inoculation (n=9). There is no significant difference in plant height **(a)** before disease challenge (p=0.068) or **(b)** after disease challenge (p=0.383), or **(c)** in the difference in height (p=0.924) between the receiver and donor plants. Positive number indicates an increase in height and a negative number indicates a decrease in height.

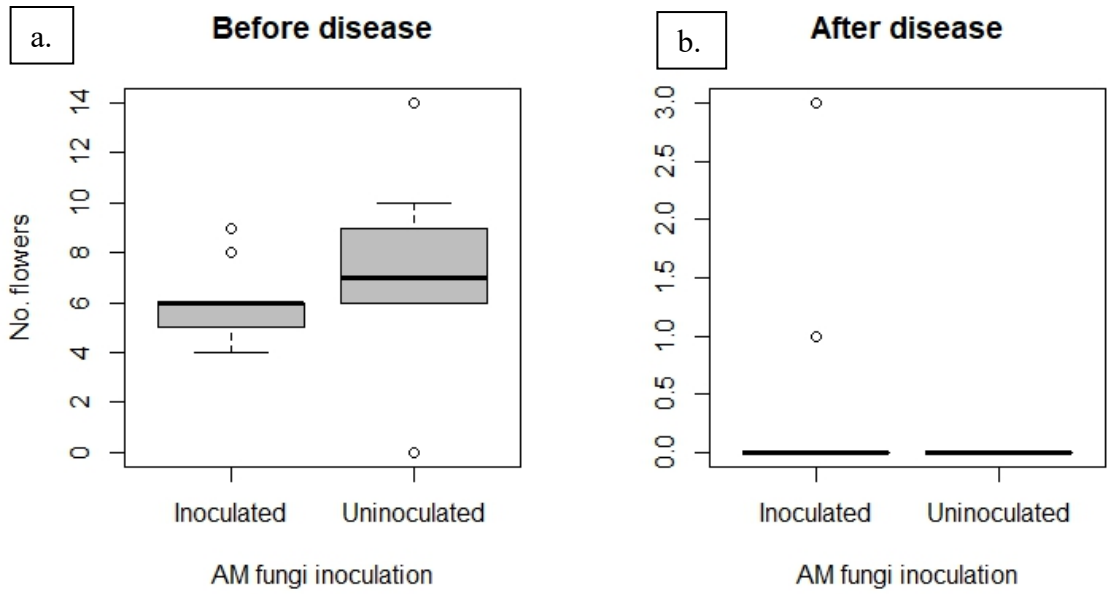


Figure 18. Boxplot for chickpea plant number of flowers before disease and after disease based on AM fungi inoculation (n=9) or no inoculation (n=9). There was no significant difference between the number of flowers in **(a)** plants before disease (p=0.385) or **(b)** after disease (p=0.17).

3.4.5 Effect of AM fungi inoculation on plant biomass

A two-sample t-test was conducted on chickpea plant biomass to determine if there was any effect of AM fungi inoculation. The results indicated that there was no significant difference ($t(16)=1.15$, $p=0.134$, $d=0.073$; Fig. 19) in biomass between inoculated plants ($\bar{x}=2.35$ g) and uninoculated plants ($\bar{x}=2.07$ g). Sample C12 is shown as an outlier in the uninoculated treatment at 1.19 g.

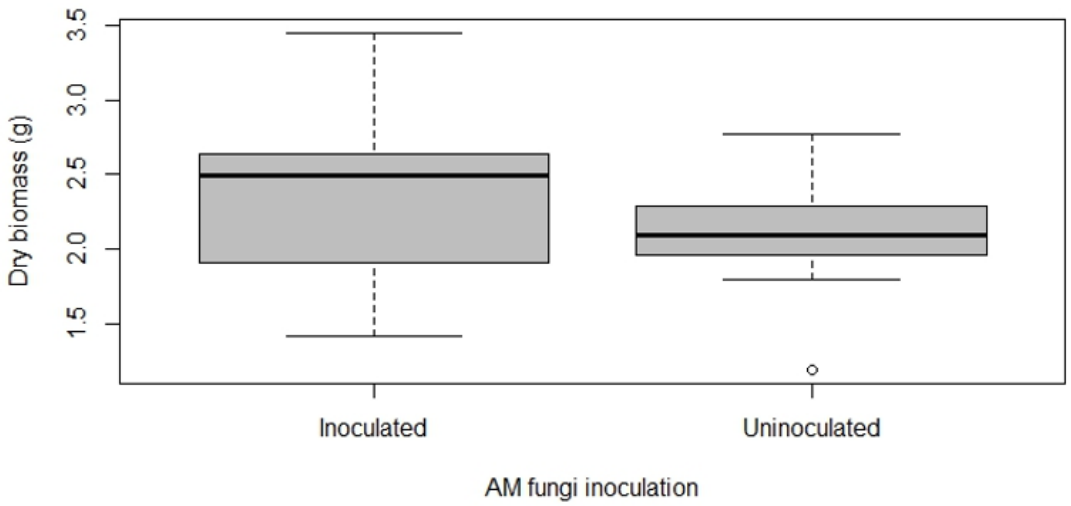


Figure 19. Boxplot for chickpea plant above-ground dry biomass based on AM fungi inoculation (n=9) or no inoculation (n=9). There was no significant difference in biomass between plants that were inoculated with AM fungi and those that were not (p=0.134).

3.5 DISCUSSION

This greenhouse experiment demonstrated a significant decrease in disease severity in chickpea after challenge with AB in plants inoculated with AM fungi. This is the first known study to show these results in chickpea plants challenged with *A. rabiei* in potting mix in a greenhouse.

The hypothesis that AM fungi would decrease disease severity in inoculated plants was supported (Fig. 16). Inoculated plants were found to have a disease rating of seven whereas uninoculated plants were found to have a disease rating of eight. Although this has no real-world application at this time, it paves the way for future research in the potential of AM fungi as a disease reduction mechanism in chickpea AB.

Plant nutrition is improved by AM fungi by providing key minerals that support the creation of photosynthetic structures and thereby chlorophyll content, enhancing the plant's ability to defend itself against pathogens (Weng *et al.,* 2022b). Potential mechanisms of

disease reduction that may be supported by AM fungi symbiosis in chickpea include the production of barrier chemicals such as lignin and phytoalexins (Thakur *et al.,* 2023) and supporting plant synthesis of key defense hormones including salicylic acid and jasmonic acid (JA) (Ruan *et al.,* 2019; Weng *et al.,* 2022b). Arbuscular mycorrhizal fungi have been a mechanism of disease reduction against other pathogenic fungi, specifically for tomato plants that were challenged with *Botrytis cinerea* when inoculated with *Gigaspora margarita* AM fungi (Fujita *et al.,* 2022). These plants mounted a faster and stronger defense response than those that were not inoculated. In addition, the AM fungi symbiosis is also effective against tomato early blight caused by the pathogenic fungus *Alternaria solani* (Song *et al.,* 2015) and cotton plants against *Verticillium dahlia*, another pathogenic fungus (Goicoechea, 2020).

The hypothesis that AM fungi inoculation would increase plant height was not supported in this experiment (Fig. 17). Even though the result was not significant, in the absence of rhizobium that supply N to the plant, the inoculated treatment was on average two centimetres taller than controls. Typically, both rhizobium and AM fungi will create a symbiosis through the same signalling pathway secreting lipo-chitooligosaccharides to enter the roots (Wang *et al.,* 2022). The dual symbiotic relationship of rhizobium and AM fungi in chickpea roots may contribute to the increase in plant height through increased uptake of nutrients, especially N and P content in this legume (Mohammadi *et al.,* 2011) so it is interesting that an increase was still observed in the absence of rhizobium. In a study on a prairie legume (*Amorpha canescens,* "lead plant"), symbiosis with both species increased plant biomass and root nodule number but in the presence of low N, nodulation was decreased (Larimer *et al.,* 2014). Perhaps the AAFC potting mix was too low in N so the plant created a symbiosis with only AM fungi rather than both the fungi and rhizobium

(Larimer *et al.,* 2014; Tariq & Ahmed, 2023). In support of this experiment's results, research conducted with intercropped faba bean (a legume) and wheat with an AM fungi mixture in pots, produced faba bean that was not significantly different in height than the wheat, possibly due to a reduction in N supply to the faba bean (Ingraffia *et al.,* 2019).

In a single-root pot experiment, a triple AM fungi species mix (*F. mosseae, R. intraradices, Glomus claroideum*) was applied to leek plants that resulted in an increased supply of plant available P and subsequently an increase in plant height – attributed to functional complementarity in the AM fungi (Jansa *et al.,* 2008). In agricultural fields, there are multiple species of AM fungi, and this experiment replicated these conditions by applying a dual species commercial AM fungi inoculant (*F. mosseae, R. intraradices*). The ability of AM fungi to supply P to the roots lies in their phosphate transporters like *GvPT* using proton-coupled symport in the extra-radical mycelia (ERM), identified in both *F. mosseae* and *R. intraradices* (Ferrol *et al.,* 2019).

The hypothesis that the number of flowers on the chickpea plants inoculated with AM fungi would be higher than uninoculated plants was not supported (Fig. 18); however, prior to disease challenge plants produced more flowers. Rather than contributing to increased biomass, the AM fungi symbiosis may be beneficial to the chickpea in the production of more flowers and therefore set more seed – a positive result for seeing increased yields. In two of the inoculated chickpeas, even after disease developed in the plants, sample C6 still had three viable flowers and sample C2 had one. In the presence of disease, AM fungi can provide damage compensation – the ability to regrow after stress or damage is encountered – that may have occurred in this experiment.

The hypothesis that chickpea above-ground dry biomass would be significantly higher in inoculated plants was not supported (Fig. 19). The range of biomass was larger within

the AM fungi treatments (2 g) versus the controls (0.5 g) suggesting that AM fungi have a varied result in chickpea biomass. The non-significant result is generally in contrast to the literature that for example showed a 61.6% increase in biomass in faba bean (legume) when inoculated with AM fungi in a microcosm design using nylon mesh (Qiao *et al.,* 2016) and an increase in chickpea biomass (Li *et al.,* 2022).

3.4.1 Constraints

Agricultural soils are typically more complex with a diversity of microbial organisms depending on farm management practices, and the potting mix used in these experiments would not have the same diversity of organisms. This could exclude important interspecies interactions between the plant, AM fungi, and rhizobium that would normally occur in the field. Chickpeas were covered with Sun bags after conidial spore application to increase humidity and prevent contamination of the controls, but this prevented watering the top of the soil and water was applied directly to the saucer. The bags may also have contributed to restriction of plant height. The small size of the H-pots caused root binding that may have limited plant growth and contributed to plant stress. Since rhizobium symbiosis did not occur, this may have constrained some aspect of the chickpea's ability to fight disease that may have resulted in an even lower disease severity in inoculated treatments.

3.5.2 Future Research

To follow-up on these results, future research can include repeating this experiment with a different potting mix or soil mixture to ensure rhizobium symbiosis occurs. Applying disease pressure at different conidial spore concentrations and at different chickpea growth stages might yield interesting results. Removal of the Sun bag may also result in lower levels of disease but may better mimic field conditions that are typically not that humid. Determining the best chickpea variety and AM fungi combination for maximum disease

reduction could allow producers to choose what works best for them and their particular farm management approach. Conducting field trials using natural soil AM fungi communities and varying levels of disease pressure to assess the real-world application potential of this symbiosis is a crucial step in future research for AB disease management.

3.5.3 Conclusion

Arbuscular mycorrhizal fungi inoculation reduced AB disease severity in chickpeas grown in a greenhouse in a soilless medium. Possible mechanisms of disease reduction include activating systemic disease resistance pathways and improving antioxidant production while increasing expression of key genes involved in defense signaling. Fungicides are typically used as the main disease management method; however, these can pose a risk to environmental and human health (Baćmaga *et al.,* 2016; Belsky & Joshi, 2020). When foliar fungicides are applied at appropriate times and doses, AM fungi propagules in the soil can remain viable (Fanning *et al.,* 2022b; Okiobe *et al.,* 2022) providing support during disease pressure. The responsible use of fungicides and implementation of farm management practices that support AM fungi populations may be beneficial in managing AB. These results are important for future research on the chickpea-AB pathosystem as *A. rabiei* poses a risk for evolving increased aggressiveness and insensitivity against single MoA fungicides.

CHAPTER 4: CONCLUDING STATEMENT

New methods of disease control for AB of chickpea crops are required. AM fungi have shown promise in reducing disease severity (Dey & Ghosh, 2022; Schouteden *et al.,* 2015; Weng *et al.,* 2022b; Whipps, 2004). These results showed that AM fungi can significantly reduce AB disease severity in chickpeas. This study's findings are an important preliminary investigation into AM fungi as a complementary disease management tool in conjunction with appropriate use of fungicides. As our growing population relies on sustainable sources of nutrition, chickpeas become an even more important crop. By nurturing the AM fungi symbiosis in conjunction with a sustainable integrated pest management plan, chickpea producers may increase their yields by decreasing disease severity, increase profits, save money on fungicides, and improve soil health.

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APPENDIX A: ROOT AM FUNGI COLONIZATION ESTIMATION

** **Root colonization** AM fungi structures dyed with Trypan blue identified using a light microscope at 40x magnification (hyphae, vesicles, spores) (Fig. 13).

INTERCROP EXPERIMENT: CHICKPEA ROOT COLONIZATION ESTIMATION

SAMPLE	*TREATMENT*	*ROOT 1*	*ROOT 2*	*ROOT 3*	*ROOT 4*	*TOTAL*
A244	BLK	15	15	15	20	65%
L230	BLK	25	20	25	15	85%
M232	BLK	25	20	25	20	90%
K233	BLK	25	25	25	25	100%
G196	CMN	25	5	25	10	65%
E188	CMN	20	20	15	20	75%
B52	CMN	25	25	25	25	100%
D151	NM	25	20	25	10	80%
F2	NM	25	20	25	25	95%
						84%

INTERCROP EXPERIMENT: FLAX ROOT COLONIZATION ESTIMATION

SAMPLE	*TREATMENT*	*ROOT 1*	*ROOT 2*	*ROOT 3*	*ROOT 4*	*TOTAL*
D111	BLK	25	25	10	15	75%
H106	BLK	25	25	20	20	90%
E203	CMN	10	25	25	20	80%
G196	CMN	25	25	25	20	95%
F200	NM	15	20	25	15	75%
M154	NM	20	20	15	25	80%
L160	NM	15	20	25	20	80%
B21	NM	25	25	15	20	85%
A157	NM	25	25	25	20	95%
						84%

CHICKPEA DONOR-RECEIVER EXPERIMENT: ROOT COLONIZATION ESTIMATION

SAMPLE	*MESH TYPE*	*ROOT 1*	*ROOT 2*	*ROOT 3*	*ROOT 4*	*TOTAL*
1015	CMN	20	25	25	25	95%
1002	CMN	25	25	25	20	95%
1004	CMN	25	20	20	20	85%
2006	BLK	20	15	15	15	65%
2012	BLK	25	20	20	20	85%
2009	BLK	20	20	25	20	85%

CMN TOTAL: 92%
BLK TOTAL: 78%

APPENDIX B: AAFC SOILLESS MEDIUM INGREDIENTS

Nutrient content of the soilless growing medium:

Calcium	1709 mg/litre	Fluoride	7.1 mg/litre
Phosphorous	958 mg/litre	Zinc	6.1 mg/litre
Nitrogen	756 mg/litre	Manganese	4.5 mg/litre
Potassium	505 mg/litre	Copper	2.24 mg/litre
EDTA	41.9 mg/litre	Boron	0.9 mg/litre
Magnesium	28.0 mg/litre	Molybdenum	0.03 mg/litre
Iron	16.7 mg/litre		
Total litres of mix	357 litres		

APPENDIX C: PRO-MIX® POTTING MIX INGREDIENTS (PROPRIETARY)

INGREDIENTS

- Canadian sphagnum peat moss (60-75%)
- Peat humus (except 2 cu ft comp.)
- Compost (except 2 cu ft comp.)
- Perlite
- Gypsum
- Limestone (for pH adjustment)
- Organic fertilizer
- Mycorrhizae - PTB297 Technology
- Coir / coconut fibre (2 cu ft comp. only)