Evaluation of *Rhizobium* spp. as effective biocontrol agents for Aphanomyces root rot in field pea (*Pisum sativum* L.)

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ABSTRACT

Aphanomyces root rot (ARR), caused by the soil-dwelling oomycete *Aphanomyces euteiches*, poses a significant threat to global pulse crop production, causing severe root damage and yield reductions in field pea and other legume crops. Given the limited effectiveness of traditional disease management methods, this study explored the biocontrol potential of *Rhizobium* spp., dinitrogen (N_2) fixing bacteria known for their symbiotic relationship with legume roots and use as N fertilizer replacements.

Obtained in this study from the nodules of *Pisum sativum* L. trap plants, a total of 70 bacterial isolates were screened for antagonistic activity towards *A. euteiches* in dual plate bioassays. An additional 24 isolates from existing culture collections and field collected legume nodules were also subjected to these assays. Most isolates, which were identified as rhizobia, were non-antagonistic. In comparison, three non-rhizobial nodule endophytes (NREs) isolated from the nodules of *P. sativum* trap plants— *Burkholderia* strain SU2B2, *Kosakonia cowanii* LL3B1, and *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 — exhibited antagonistic activity towards *A. euteiches in vitro*. Notably, *K. cowanii* LL3B1 was capable of completely halting *A. euteiches* colony growth for over 30 days.

In three sets of completely randomized sterile growth pouch assays, NREs *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2, when applied as liquid seed inoculants, demonstrated the potential to enhance *P. sativum* nodulation by *Rhizobium* strains and reduce disease severity without affecting initial leaf development. Additionally, they increased N_2-fixation in *P. sativum* compared with uninoculated controls.

This study also revealed the diversity of *Rhizobium* associated with *P. sativum* nodules, by isolating and identifying seven distinct species within the Rhizobiaceae family from *P.*

sativum nodules. While none showed independent antagonism towards *A. euteiches*, the presence of certain Rhizobiaceae species alongside *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 showed potential to synergistically enhance antagonistic activity towards *A. euteiches in vitro* when evaluated in dual plate bioassays. Additional research is recommended to assess their impact on plant health and potential synergistic effects when applied as seed inoculants along with rhizobia.

These findings indicate that NREs, particularly *K. cowanii* and *P. chlororaphis* subsp. *aurantiaca*, hold potential as biocontrol agents against ARR. Consequently, further investigation of the biocontrol capabilities of bacteria associated with legume nodules may improve the efficacy of current disease mitigation strategies.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Aphanomyces root rot (ARR) is a global threat to pulse crop production caused by the oomycete *Aphanomyces euteiches*, a water mold belonging to the class Oomycota (Wu et al., 2018). It is known to infect many different legumes, most notably field pea (*Pisum sativum* L.) and lentil (*Lens culinaris*), as well as other legumes including alfalfa, faba bean, red kidney bean, snap bean, red clover, and white clover (Papavizas & Ayers, 1974). It can cause severe root damage at all growth stages of its host and when root rot is severe, yield reduction can be as high as 70% (Wu et al., 2018). Traditional management methods for plant diseases, such as fungicide application or crop rotation, have been minimally effective at reducing or eradicating ARR (Wu et al., 2018).

Alongside ARR, *A. euteiches* infection co-occurs with multiple soil-borne fungi and oomycete pathogens in the pea root rot complex (PRRC) in *P. sativum* (Wille et al., 2021). Fungi and oomycete pathogens in the PRRC can include *A. euteiches*, *Didymella pinodella*, *Fusarium avenaceum*, *Fusarium oxysporum* f. sp. *pisi*., *Fusarium redolens*, *Fusarium solani* f. sp. *pisi*, *Pythium* spp., *Phytophthora* spp., and *Rhizoctonia solani* (Xue, 2003; Wu et al., 2018, Wille et al., 2021).

Field pea (*Pisum sativum* L.) is one of many pulse crops. Pulse crops contain relatively high quantities of protein compared to cereals and produce all 20 amino acids, 9 of which humans cannot produce on their own, and hence are a valuable component of healthy food systems (Robinson et al., 2019). The worldwide average per day per capita consumption of pulses is 21 g (Rawal & Navarro, 2019). In 2022, 6.5 M tonnes of pulse crops were produced in Canada (Agriculture and Agri-Food Canada, 2023). Of the total pulse crop production in Canada in 2022,

3.4 M tonnes were dry peas (Agriculture and Agri-Food Canada, 2023). Comparatively, global pea production was approximately 14.9 M tonnes in 2020 (Boersch, 2022).

Field pea forms a symbiotic relationship with atmospheric dinitrogen (N_2) fixing bacteria known as *Rhizobium* spp. These rhizobia induce the formation of nodules on the root system where the nitrogenase enzyme reduces N_2 and makes it available to the plant as well as the soil system, enhancing subsequent crop yields (Clayton et al., 2004). For example, Knight (2016) reported that successful inoculation of faba bean (*Vicia faba* L.) doubled the percentage of nitrogen (N) from fixation and increased biomass by 33%. This symbiosis is especially important in developing countries due to high cost and availability of N fertilizers. Inoculation of field peas with rhizobia can increase seed yield and improve yield stability in western Canada. Inoculation can increase seed protein yields by up to 15% (Clayton et al., 2004).

Recently, a diverse set of soil-borne bacteria including two *Rhizobium lemnae* strains demonstrated antagonistic activity towards *A. euteiches in vitro a*nd *in planta* (Godebo et al., 2020). Because rhizobia form a symbiosis with legumes to fix N_2 and are applied as commercial inoculants to replace N fertilizer, there is potential for select strains to be used as biocontrol agents for legume pathogens. Successful development of a rhizobial symbiotic inoculant to control *A. euteiches* could both strengthen global food security and agriculture sustainability while reducing disease management costs.

1.1 Project Objectives and Hypotheses

The overall objective of this project was to isolate rhizobia which when applied as inoculants will both nodulate field pea and suppress Aphanomyces root rot, enhancing overall crop resilience and sustainability.

The specific hypothesis of this research was:

There are *Rhizobium* spp. isolates which will nodulate field pea (*P. sativum*) and limit Aphanomyces root rot severity in their host.

Two key questions associated with this hypothesis were:

Are *Rhizobium* spp. generally antagonistic towards Aphanomyces root rot?

Are *Rhizobium* spp. a feasible biocontrol option?

Specific objectives of this project were:

1. Isolate a diverse set of bacteria associated with *P. sativum* nodules;
2. Evaluate these bacteria for inhibition of *A. euteiches* mycelial growth;
3. Determine the identity of these bacteria using molecular techniques;
4. Evaluate the effects of candidate biocontrol bacteria on *P. sativum* nodulation, health, and root rot disease outcomes when applied as a liquid inoculant in growth pouch experiments.

2 LITERATURE REVIEW

2.1 Biological control of Aphanomyces root rot

Biological control, also known as biocontrol, can be generally described as a process by which an organism limits the growth or propagation of another, namely undesired pathogens (Gopalakrishnan et al., 2015). In the case of fungal pathogens, there are three distinguishable strategies for limiting growth based on the life cycle of the fungal pathogen. The first strategy relies on microbial protection of a host against infection; the second being microbial interference with colonisation and sporulation of a pathogen; and the third being microbial interference with the surviving structures of a pathogen (Fokkema, 1993). Accordingly, there are four main target phases of these strategies, according to the life cycle of the pathogen involved (Fig. 2.1).

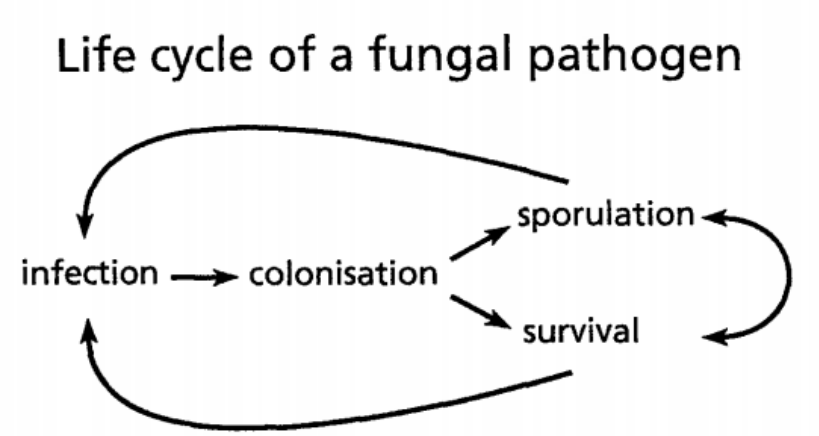

Figure 2.1 Life cycle of a fungal pathogen, showing the four main target phases of a biological control agent. Figure adapted from Fokkema (1996).

Certain soils, known as suppressive soils, are less susceptible to specific crop diseases (Schlatter et al., 2017). This ability is likely a result of the presence of a specific community of beneficial indigenous microorganisms inhabiting the respective soil, which limits the growth of the potential pathogen. Most notably, these include organisms belonging to the order *Bacillales*, and genera *Pseudomonas* spp., *Trichoderma* spp., and *Streptomyces* spp. (Schlatter et al., 2017).

Consequently, this has led to the idea of harnessing rhizosphere microorganisms for pathogen and pest biocontrol (Ciancio et al., 2019). As a phytopathogen management strategy, biological control can be advantageous as it is often low cost and sustainable, while boasting a number of options specific to individual needs (Syed Ab Rahman et al., 2018).

At the present moment, Aphanomyces root rot (ARR) does not respond well to typical plant pathogen management methods, such as fungicide application (Wu et al., 2018). Consequently, the best-known method to control the disease is to avoid cultivating legumes in infected fields for approximately 10 years, due to the lengthy viability of *A. euteiches* oospores (Gaulin et al., 2007). There is some evidence suggesting that a pre-emergence herbicide treatment consisting of a combination of glyphosate, sulfentrazone and trifluralin can reduce root rot disease severity in *P. sativum* (Asare et al., 2022). Due to limited success with conventional disease management practices, biological control strategies may provide another option for future management of ARR.

A recent meta-analysis of literature surrounding the biological control of ARR has found that there is potential for biological control of ARR and recommended that more field trials be conducted (Godebo et al., 2022). Additionally, it was found that there is a lack of detail surrounding the mechanisms of biological control of ARR within these studies (Godebo et al., 2022).

There are multiple known isolates with demonstrated *in vitro* antagonism towards *A. euteiches*. An isolate of *Bacillus cereus* significantly reduced visual root rot disease in an initial trial (Wakelin et al., 2002). Another bacterium, *Paenibibacillus polymyxa*, was the most effective at reducing oospores in *P. sativum* roots, with a reduction from 450 to 160 g^{-1} of root (Wakelin et al., 2002). Different bacteria have been tested as seed treatments, and showed success in trials with other root diseases, such as take-all of wheat (Wakelin et al., 2002). For example, Wakelin et al.

(2002) reported that of 558 bacterial isolates recovered directly from soil samples, three isolates produced zones of inhibition against *A. euteiches* mycelium in dual-culture bioassays. Of 146 bacterial isolates recovered from the rhizosphere region of *P. sativum*, 28 were able to variably suppress the *in vitro* growth of *A. euteiches* mycelium (Wakelin et al., 2002). Of these antagonistic isolates, all were able to reduce zoospore germination of *A. euteiches in vitro*, and of these 28, seven were inhibitory by more than 50% relative to controls (Wakelin et al., 2002).

More recently, a screening of bacterial isolates obtained from soil samples across Saskatchewan and from culture collections identified 184 isolates which were antagonistic towards *A. euteiches* (Godebo et al., 2020). Most of these isolates were *Bacillus* spp., *Pseudomonas* spp., *Paenibacillus* spp., *Lysobacter* spp., and *Streptomyces spp.* Intriguingly, three isolates were identified as *Rhizobium* spp., which presented the possibility of developing disease-suppressant rhizobia-based inoculants given further study (Godebo et al., 2020).

2.2 *Aphanomyces euteiches*

2.2.1 Ecology of *Aphanomyces euteiches*

Aphanomyces euteiches is an oomycete, which are fungal-like eukaryotes belonging to the kingdom Chromista (Beakes et al., 2012). There are two main disease-causing orders of oomycetes, Saprolegniales (*Aphanomyces* spp.) and Peronosporales (containing notable members such as *Pythium* spp. and *Phytophthora* spp.) (Sharma et al., 2021). These oomycetes produce what are known as zoospores, which are motile and swim towards plant surfaces where they then chemotactically or electroctactically attach themselves (Tyler, 2002). Here they shed their flagella, encyst, then germinate forming a germ tube which develops an appressorium (Sharma et al., 2021). The appressoria generally function to penetrate the cells of the plant host. Despite being an oomycete, *A. euteiches* does not form distinct appressoria (Kemen & Jones, 2012). Instead, *A.*

euteiches produces a zoosporangium which form coenocytic hyphae that penetrate intracellular spaces of the root cortex (Badreddine et al., 2008). Infection can occur at any growth stage of the plants and spreads rapidly with high soil moisture and temperatures from 22 to 28°C (Burke et al., 1969).

The life cycle of *A. euteiches* has both asexual and sexual reproduction stages involving the succession of oospore and zoospore formation (Papavizas & Ayers, 1974; Laloum et al., 2021). Sexual oospores can be readily seen in the root tissue with a compound microscope (Papavizas & Ayers, 1974). The oospores of *A. euteiches* are particularly hardy and can survive in the soil for more than ten years (Papavizas & Ayers, 1974).

2.3 Biochemistry of *Aphanomyces euteiches*

Oomycetes are cellulosic fungus-like microorganisms which typically lack chitin in their cell walls (Badreddine et al., 2008). Analysis of *A. euteiches* cell walls showed they consisted of 10% N-acetyl-D-glucosamine and contained noncrystalline chitosaccharides necessary for cell wall integrity (Badreddine et al., 2008). These noncrystalline chitosaccharides are exposed at the cell wall surface, as opposed to normal crystalline chitin which is usually embedded in inner cell wall layers (Badreddine et al., 2008).

2.4 *Rhizobium* spp.

2.4.1 Ecology of *Rhizobium* spp.

Rhizobia are a vital bioresource due to their ecological and economic impacts (Wang et al., 2019). Relevant ecological problems, such as climate change and environmental degradation caused by overuse of chemical fertilizers call for more study into the ecology and application of rhizobia (Gopalakrishnan et al., 2015; Wang et al., 2019). Rhizobia typically inhabit the root nodules of legumes, which can host multiple distinct species of rhizobia (Johnston & Beringer, 1976).

Rhizobia harness both direct and indirect plant growth promotion (PGP) traits (Gopalakrishnan et al., 2015). Direct traits primarily include atmospheric N_2 fixation (i.e., biological nitrogen fixation (BNF)), and thus rhizobia can be applied as legume inoculants to replace N fertilizers in agricultural systems. Direct PGP traits also include phosphate solubilization, siderophore formation, and phytohormone production (Gopalakrishnan et al., 2015). Indirect traits include the induction of systemic resistance against phytopathogens and pests. This can take form as production of antibiotics, secretion of siderophores depriving fungal pathogens of iron uptake, production of low molecular weight metabolites such as hydrocyanic acid (HCN), production of lytic enzymes such as chitinase, β-1,3 glucanase, protease and lipase, competition for nutrients against phytopathogens, and induction of systemic resistance in plants (Reddy, 2013).

2.4.2 Biocontrol of phytopathogens with *Rhizobium* spp.

Certain rhizobia possess biological control properties towards phytopathogens (Table 2.1), such as *Fusarium*, *Rhizoctonia*, *Sclerotium*, and *Macrophomina* (Das et al., 2017; Kebede, 2021). In addition, two different *Rhizobium lemnae* strains are antagonistic towards *A. euteiches in vitro* (Godebo et al., 2020). Strains of rhizobia including *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, *R. meliloti*, *R. trifolii*, *S. meliloti* and *B. japonicum* produce antibiotics and cell-wall degrading enzymes that can inhibit some phytopathogens (Ozkoc & Deliveli, 2001; Bardin et al., 2004; Chandra et al., 2007). Known mechanisms for biological control include competition for nutrients, production of antibiotics, cell wall degrading enzymes, siderophores, and secondary metabolites such as phenazines (Bhattacharrya & Jha, 2012; Gopalakrishnan et al., 2015).

Table 2.1 The fungicidal spectrum of *Rhizobium* spp. isolates towards various plant pathogens and their host crop (adapted from Das et al., 2017).

Organism	Plant pathogen	Host	Reference
Rhizobium sp. NBRI9513	*Fusarium* spp. *Rhizoctonia bataticola* *Pythium* sp.	Chickpea	Nautiyal (1997)
Rhizobium sp. *Bradyrhizobium* sp.	*Macrophomina phaseolina*, *Rhizoctonia solani*	Soybean, Sunflower, Okra	Omar and Abd-Alla (1998); Siddiqui et al. (2000)
Rhizobium meliloti	*Macrophomina phaseolina*	Ground nut	Arora et al. (2001)
Bradyrhizobium japonicum	*Macrophomina phaseolina*, *Fusarium solani;Rhizoctonia solani*	Tomato	Siddiqui and Shaukat (2002)
Rhizobium sp.	*Fusarium solani* f. sp. *phaseoli*	Bean	Estevez de Jensen et al. (2002)
Rhizobium sp.	*Fusarium oxysporum* f. sp. *ciceri*	Chickpea	Arfaoui et al. (2005)
Mesorhizobium loti MP6	*Sclerotinia sclerotiorum*	Indian mustard	Chandra et al. (2007)
Rhizobium Leguminosarum bv. *viceae*	*Pythium* spp.	Pea, lentil	Huang and Erickson (2007)
Rhizobium sp.	*Sclerotium rolfsii*	Ground nut	Ganesan et al. (2007)
*Sinorhizobium fredii*KCC5	*Fusarium udum*	Pigeon pea	Kumar et al. (2010)
Rhizobium leguminosorum	*Fusarium oxysporum* f. sp. *ciceri*	Chickpea	Singh et al. (2010)
Ensifer meliloti, Rhizobium leguminosarum	*Fusarium oxysporum*	Fenugreek	Kumar et al. (2011)
Rhizobium sp.	*Fusarium oxysporum*, *Fusarium solani*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotium rolfsii*	Faba bean, Chickpea, Lupine	Shaban and El-Bramawy (2011)
Rhizobium sp.	*Cylindrocladium parasiticum*	Soybean	Gao et al. (2012)
Rhizobium japonicum	*Fusarium solani*, *Macrophomina phaseolina*	Soybean	Al-Ani et al. (2012)
Rhizobium sp. RS12	*Fusarium solani*, *Macrophomina phaseolina*	Chickpea	Smitha and Singh (2014)

2.5 Biocontrol of Phytopathogens by Select Bacteria Other Than Rhizobia

2.5.1 *Kosakonia cowanii* classification and swarming

Kosakonia cowanii is a motile, gram-negative, facultatively anaerobic and rod-shaped bacterium (Han et al., 2023). Before the year 2000, *Kosakonia cowanii* was taxonomically classified as part of *Enterobacter agglomerans* and *Pantoea agglomerans* (Berinson et al., 2020). In the year 2000, it was reclassified as *Enterobacter cowanii*. Most recently, in 2013 the bacterium was further classified as *Kosakonia cowanii* (Brady et al., 2013).

2.5.2 *Kosakonia cowanii* swarming and exopolysaccharide production

Swarming bacteria are often accompanied by slime production associated with gliding motility and are correlated with the presence of flagella (Williams and Schwarzhoff, 1978; Harshey, 2003). *Enterobacter* strain SM1 and SM3 swarm radially on 0.5 % Luria Broth (LB) agar plates (Chen et al., 2021). *Kosakonia radicincitans* DSM 16656 is motile and carries genes for two flagellar systems and three type VI secretion systems (Becker et al., 2018). Exopolysaccharides (EPS) are extracellular macromolecules excreted as a slime layer in microorganisms (Angelin & Kavitha, 2020). *Kosakonia cowanii* LT-1 produces exopolysaccharides (Gao et al., 2020). Additionally, polysaccharides and surfactants are components of what is considered slime produced by surface colonies (Toguchi et al., 2000). Exopolysaccharides and surfactants in slime offer protection from desiccation by water retention (Ophir & Gutnick, 1994).

2.5.3 *Kosakonia cowanii* biocontrol

Kosakonia cowanii produces cellulase and chitinase and was found to be highly antagonistic toward *Cercospora* spp. (Lirio et al., 2020). *Enterobacter cowanii* operates as a biocontrol agent for *Fusarium verticillioides*, *Alternaria tenuissima*, and *Botrytis cenerea* where it effectively inhibits *Botrytis cenerea* phytopathogen of tomato *in vivo* (Shi & Sun, 2017). Additionally, *K. cowanii* can treat citrus canker in seedling and adult navel oranges caused by *Xanthomonas citri* subsp. *citri* (Lai et al., 2022). *Enterobacter cowanii* also demonstrates good performance in biological control of crown gall disease caused by *Agrobacterium tumefaciens* (Li et al., 2017).

2.5.4 *Kosakonia cowanii* plant growth promoting benefits

Kosakonia cowanii is a non-rhizobial bacteria showing plant growth-promoting bacteria (PGPB) activity, including increasing number of nodules per plant, root and shoot dry weights, plant, shoot and root N concentration in alfalfa (Noori et al., 2018). Bacteria belonging to the genus *Enterobacter* can be isolated from nodules of *P. sativum* (Tariq et al., 2014). Despite being non-nodulating, they can fix N_2 when evaluated in an acetylene reduction assay (Tariq et al., 2014). Moreover, they can solubilize phosphorus and colonize the root of *P. sativum* when re-inoculated (Tariq et al., 2014). Additionally, two *Enterobacter* spp. strains provided N to sugarcane plants via BNF and promoted sugarcane growth under gnotobiotic conditions (Lin et al., 2012). A *K. cowanii* strain isolated from the wheat rhizosphere also demonstrated PGP on Pak choi (*Brassica rapa* subsp. *chinensis*) (Wang et al., 2022). Another *K. cowanii* strain promoted the seed germination rate and growth vigor of maize (Gao et al., 2020). To compliment these PGP traits, the application of *K. cowanii* mitigates drought conditions in *Arabidopsis thaliana* (Jeong et al., 2021).

Despite the aforementioned benefits in plants, not all *K. cowanii* strains are considered beneficial. In Patchouli (*Pogostemon cablin*), *K. cowanii* Pa82 is identified as the causal agent of bacterial wilt (Zhang et al., 2022). Additionally, a species of *K. cowanii* is a causal agent of bacterial blight in soybean (*Glycine max* L.) (Krawczyk & Borodynko-Filas, 2020), and stalk rot in foxtail millet (*Setaria italica*) (Han et al., 2023). Furthermore, there are concerns that *K. cowanii* can be a human pathogen. According to Berinson et al. (2020), *K. cowanii* 888-76 can be a rare cause of acute cholecystitis. However, an Environmental Protection Agency (EPA) exemption from the requirement of a tolerance for *K. cowanii* SYM00028 residues in or on all food commodities is currently instated (Environmental Protection Agency, 2021).

2.5.5 *Pseudomonas chlororaphis* subsp. *aurantiaca* biological control

Pseudomonas chlororaphis subsp. *aurantiaca* produces phenazine compounds (Hu et al., 2014; Morohoshi et al., 2017; Mehnaz et al., 2020; Liu et al., 2022). These biomolecules are known for broad-spectrum antifungal activity by direct fungal suppression of important agricultural phytopathogens such as *Fusarium* spp. and *Pythium* spp. (Tomashow & Weller, 1996). *Pseudomonas chlororaphis* subsp. *aurantiaca* PB-St2 and StFRB508 produce acyl homoserine lactones (AHL) (Bauer et al., 2016; Morohoshi et al., 2017), which are integral to the regulation of the *P. chlororaphis* subsp. *aurantiaca* quorum sensing system for production of phenazine compounds (Morohoshi et al., 2017).

When grown on Luria-Bertani (LB) agar plates, phenazine production from *P. chlororaphis* subsp. *aurantiaca* can be categorized visually into three levels: (1) high production (deep orange with diffusion); (2) low production (moderate orange without diffusion); and (3) no production (white or very pale yellow) (Morohoshi et al., 2017). Maximum phenazine-1-carboxylic acid (PCA) yield was obtained at 17.81 $g \cdot L^{-1}$ glucose and 11.47 $g \cdot L^{-1}$ soytone (Yuan et

al., 2008). Three kinds of phenazine compounds were produced by *P. chlororaphis* zm-1 which could inhibit the growth of *Slecrotium rolfsii*, a pathogenic fungus which causes peanut stem rot (Liu et al., 2022).

When evaluated as a biocontrol for the oomycete phytopathogen *Pythium ultimum*¸ which causes damping-off and root rot in cucumber, *P. chlororaphis* subsp. *aurantiaca* StFRB508 significantly ($P<0.05$) increased the number of surviving plants compared with uninoculated controls (Morohoshi et al., 2017). *Pseudomonas chlororaphis* subsp. *aurantiaca* Pa40 also demonstrated clear and consistent suppression of wheat sharp eyespot disease in a greenhouse experiment, comparable to chemical treatment outcomes (Jiao et al., 2013). This strain also inhibited 10 of 13 phytopathogenic fungal strains and six of eight phytopathogenic bacteria demonstrated HCN, protease and siderophore production (Jiao et al., 2013). When isolated from the soybean rhizosphere, *P. chlororaphis* subsp. *aurantiaca* demonstrated excellent *in vitro* biocontrol when evaluated against *Macrophomina phaseolina*, *Rhizoctonia solani*, *Pythium* spp., *Sclerotinia slecrotiorum*, *Sclerotium minor*, *Sclerotium rolfsii*, *Fusarium* sp., and *Alternaria* spp. (Rosas et al., 2001). *Pseudomonas chlororaphis* subsp. *aurantiaca* Pcho10 showed strong activity against *Fusarium graminearum* conidial germination, mycelial growth, and deoxynivalenol production (Hu et al., 2014). Growth chamber and field trials showed *P. chlororaphis* subsp. *aurantiaca* Pcho10 colonized the wheat head and effectively controlled disease caused by *F. graminearum*. From this, the bacterium was determined to have high potential to be developed as a biocontrol agent against *F. graminearum* (Hu et al., 2014). *Pseudomonas chlororaphis* subsp. *aurantiaca* JD37 demonstrated significant antagonistic activity against *Bipolaris maydis* and southern maize leaf blight caused by *B. maydis* (Fang et al., 2013).

2.5.6 *Pseudomonas chlororaphis* subsp. *aurantiaca* plant growth promotion benefits

Pseudomonas chlororaphis subsp. *aurantiaca* ARS-38 isolated from the rhizosphere of cotton significantly increased root and shoot dry weights in wheat seedling growth assays (Mehnaz et al., 2020). When inoculated on wheat (*Triticum aestivum L.*), *P. chlororaphis* subsp. *aurantiaca* SR1 produced a significant increase in plant height and root length in early growth stages (Carlier et al., 2008). *Pseudomonas chlororaphis* subsp. *aurantiaca* JD37 was found to effectively colonize rhizosphere soil and internal roots of maize (*Zea mays* L.) and promote maize growth (Fang et al., 2013).

3 MATERIALS AND METHODS

3.1 Source of Rhizobia

There were three sources of rhizobia used for this study: culture collection rhizobia; putative rhizobia isolates obtained from nodules of *P. sativum* as a trap plant; and nodules of field-collected legumes *Lathyrus venosus*, *Lens culinarus,* and *P. sativum*.

3.1.1 Culture collection isolates

A selection of rhizobia isolates (n=17), previously identified through fatty-acid methyl ester (FAME) analysis, was made available for this study by Dr. Jim Germida and Dr. Bobbi Helgason from the University of Saskatchewan (Table 3.1). These isolates had been stored long-term at -80°C in glycerol, some of which had been in storage since the 1980s. To ensure the cultures were alive and contamination free, isolates were first streak-plated onto yeast extract mannitol agar (YEMA) culture media to obtain single colonies.

Table 3.1 Isolate code and identification based on fatty-acid methyl ester (FAME) analysis of culture collection rhizobia included in this study.

Isolate Code*	Identification (FAME) †
IC72	*Rhizobium* sp. *cicer*
27A9	*Rhizobium leguminosarum* bv. *phaseoli*
RP202-13	*Rhizobium* sp. *cicer*
IC76	*Rhizobium* sp. *cicer*
CP92	*Rhizobium* sp. *cicer*
WPBS3644	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT632	*Rhizobium leguminosarum* bv. *phaseoli*
9030	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT899	*Rhizobium leguminosarum* bv. *phaseoli*
WPBS3622	*Rhizobium leguminosarum* bv. *phaseoli*
9039	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT7230	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT7202	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT7100	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT2560	*Rhizobium leguminosarum* bv. *phaseoli*
CIAT7136	*Rhizobium leguminosarum* bv. *phaseoli*
LPNI-2	*Rhizobium leguminosarum* bv. *viciae*

* Isolate codes were used as an identifier prior to proper identification
† Identifications determined through fatty-acid methyl ester (FAME) analysis

3.1.2 Trap plant isolates

3.1.2.1 Field collection of soils used as inoculum for *Pisum sativum* trap plants

In October 2021, soils were sampled from legume plots located near Lucky Lake (LL), Moose Jaw (MJ), Saskatoon (SPG), and Sutherland (SU) (Fig. 3.1). These locations were identified with the help of Dr. Tom Warkentin (Crop Development Center, Department of Plant Sciences, University of Saskatchewan). The sites were sampled post-harvest, having been cultivated with *P. sativum* or *L. culinarus* before harvest. The legume plots were previously inoculated with BASF Nodulator® Duo SCG, a combined granular inoculant consisting of *R. leguminosarum* 1435 and *Bacillus subtilis* BU1814, neither of which were recovered with the methods used in this study. Lack of inoculant recovery may have resulted from poor survivability in the soil, carrying implications for commercial inoculant development and application. For instance, inoculants developed elsewhere may not be adapted to every environment.

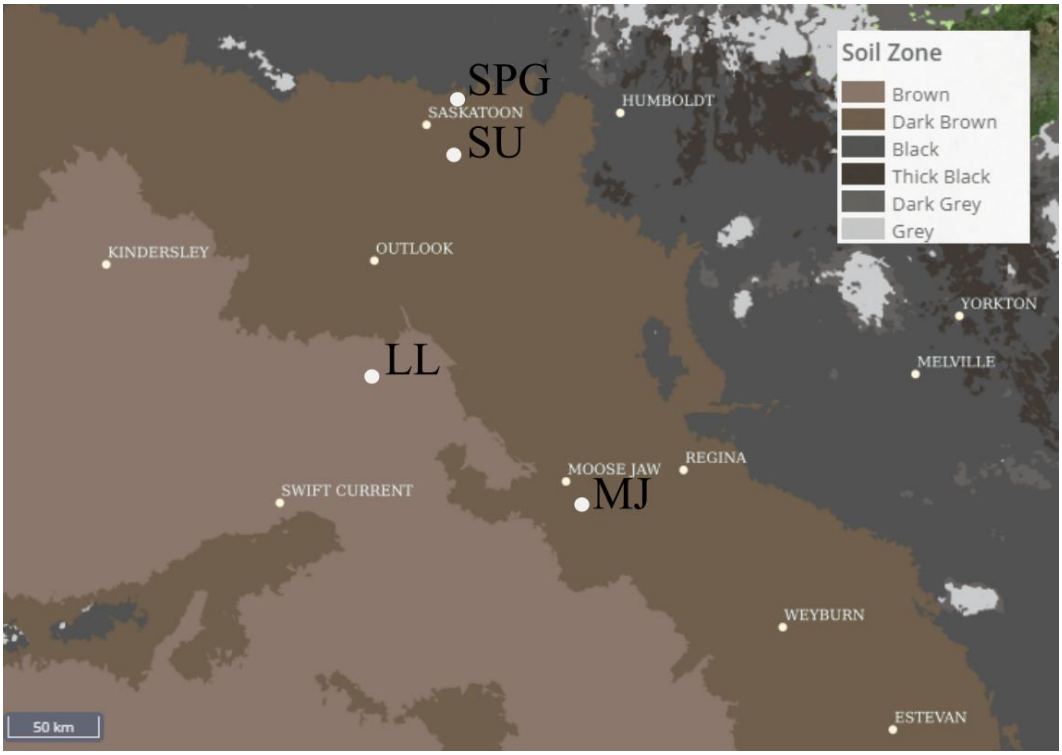

Figure 3.1 Locations of soil sampling and associated soil zones for the four legume plots (LL, MJ, SPG, and SU) in Saskatchewan, Canada, as investigated in this study.

Ten soil samples were collected at each of the four sites (for a total of 40 soil samples), with each sample being a bulk collection from three shallow holes 0 to 15 cm deep, consisting of approximately 500 g of A horizon soil. Soils were placed in plastic bags and labeled accordingly. The fields were generally flat and were randomly sampled.

3.1.2.2 Determination of field capacity

Field capacity was determined for each sampling location with two replicates. Briefly, soils were sieved through a 4 mm sieve and aggregates were broken using a mortar and pestle. After sieving, 20 cm long PVC tubes with the bottom end sealed with folded cheesecloth to allow air flow were filled with soil. Deionized water was added to the top of the tube until the top portion of the soil was visibly saturated. Parafilm was used to seal the top of the tube, and four holes were poked with scissors in the parafilm. Tubes were left at room temperature for 48 h until the wetting front stabilized. The top 1 to 2 cm of soil was then discarded, and a 20 g sample was obtained and weighed in an aluminum weigh boat. The samples were placed on a tray and dried at 80°C for 24 h in an oven. After drying, samples were reweighed, and a specific moisture content at field capacity was determined for each soil by averaging the two replicates per soil group.

3.1.2.3 Growing the trap plants in growth chamber experiment

For each soil, coffee filters were placed in the bottom of ten 10.2 cm diameter plastic pots. Pots were then filled with 500 g of air-dried soil and labeled according to the soil sample within each site (e.g., MJ10 = the 10^{th} bulked soil sample from Moose Jaw) and planting date. Soils were pre-mixed in a plastic bag with 20 mL of a modified N-free Hoagland's Solution (Appendix B). Four surface-sterilized *P. sativum* (cv. CDC Meadow) seeds were sown at a depth of approximately 2 cm in each pot. A label was placed on each pot with the calculated weight for 60% field capacity and 70% field capacity, accounting for individual pot weights. To maintain

moisture content, pots were weighed daily, and the appropriate volume of deionized water was added to the top of the pot based on the weight difference. The plants were grown in a growth chamber set to a 16 h day and 8 h night cycle, with day temperatures at 23°C and night temperatures at 21°C. Light intensity ranged from 300 to 390 $umol \cdot m^{-2} \cdot s^{-1}$ and light bulbs were Phillips T-5 Fluorescence bulb #835.

Pots were incubated in a growth chamber, covered with aluminum foil until initial leaf development, and maintained at 60% field capacity. After initial leaf development, the aluminum foil was removed, plants were thinned to two per pot, and moisture content was increased to 70% field capacity. After plants became too tall to support their own weight, they were supported using wooden stakes. Sterile techniques were used throughout the experiment when preparing, weighing, and handling soils to minimize the risk of cross-contamination.

3.1.2.4 Isolation of putative rhizobia from *Pisum sativum* trap plants

A total of 70 isolates were obtained from the nodules of *P. sativum* trap plants over the course of three harvests at 42 d, 47 d, and 63 d (Appendix A). Nodule activity is thought to be greatest during the flowering stage, and plants flowered at varying times. Accordingly, each harvest date included only plants that either flowered or were flowering. Nodules were surface-sterilized following the procedure described in Vincent (1970). Briefly, nodules were gently stirred in 70% ethanol for 3 min, transferred to a 10% sodium hypochlorite solution, then washed six times with sterile deionized water. Two methods were subsequently employed to streak plate the nodules on Congo red yeast extract mannitol agar (CRYEMA) culture media: the poke method, where the nodule was poked with a sterile pin then streak plated, and the smash method, where the nodule was pressed with sterile forceps and the juices streak plated. As Congo red absorption is generally considered a contraindication of rhizobia, colonies chosen for further isolation either did not absorb Congo Red dye or only mildly absorbed the Congo red dye. Up to two unique single colonies from each plate were transferred onto CRYEMA slants and stored in the dark at 23°C (Fig. 3.2).

Figure 3.2 A pure culture isolated from the root nodule of a *P. sativum* trap plant and stored on a CRYEMA slant.

Isolates were assigned an identifier code based on their soil (LL = Lucky Lake, MJ = Moose Jaw, SU = Sutherland, and SPG = Saskatoon), combined with a number for their associated pot (e.g., 1 = pot 1), a letter for the plant within the pot (A = plant 1, B = plant 2), and number for the unique plated single colony. For example, LL3B2 represents the 2nd unique plated single colony from a streak plated surface-sterilized nodule of the 2nd plant within the 3rd pot grown in soil obtained from Lucky Lake.

Using this system, it could be determined that isolates LL3B1 and LL3B2 were both isolated from the same nodule, while LL3A came from the other plant grown in the same pot. This identification system also allowed for further characterization of nodule-associated bacteria from trap plants grown in different soils.

3.1.3 Field collection of *Lathyrus venosus*, *Lens culinarus*, and *Pisum sativum* nodules

In early July 2021, field collections of cultivated *L. venosus, L. culinarus,* and *P. sativum* were conducted at three different fields located 60 km NE of Saskatoon, Saskatchewan, Canada. To maintain root integrity, a root ball surrounding each plant was excavated and the plants were transferred into labelled plastic bags and placed in a cooler. They were then transported to the University of Saskatchewan, where they were gently rinsed with water through a clean 1 mm sieve.

Using sterile forceps and a sterile knife, nodules were separated from the host plant and surface-sterilized following the procedure used for the trap plants (Section 3.1.2.4, p. 20). After surface-sterilization, the nodules were then transferred to a new sterile plate and sliced open with a sterile blade. Two methods were employed to streak plate the nodules on CRYEMA culture media as described above (Section 3.1.2.4, p. 20).

The plates were inverted and incubated in the dark at 23°C until single colonies formed. Distinct single colonies were purified by restreaking on 1/10 TSA plates and incubated again. Once

isolate purity was confirmed, plates were stored inverted at 4°C in the dark before being transferred to CRYEMA slants.

Lathyrus venosus nodules appeared red in the interior and were slightly larger than those of *L. culinarus*. *Pisum sativum* nodules were the largest of the three species. Red *L. venosus* nodules were streak plated using either the poke method or the smash method. *Lens culinarus* and *P. sativum* nodules with red interiors were streak plated with the poke method, while nodules with green interiors were streak plated using the smash method (Table 3.2).

Table 3.2 Putative rhizobia isolates obtained from nodules of *L. culinarus, L. venosus,* and *P. sativum* field collected specimens in 2021.

Isolate Code*	Source	Isolation Method
PP1A1	*L. venosus*	poke
PP1A2	*L. venosus*	poke
PP1A3	*L. venosus*	poke
PP2C3	*L. venosus*	poke
PP2C4	*L. venosus*	poke
PPC2	*L. venosus*	poke
PPC1	*L. venosus*	poke
PP3	*L. venosus*	smash
LIE2	*L. culinarus*	poke
L1E3	*L. culinarus*	poke
LIE4	*L. culinarus*	poke
L1SN	*L. culinarus*	poke
L2SB	*L. culinarus*	smash
LSA	*L. culinarus*	smash
FP1	*P. sativum*	poke
FP2	*P. sativum*	poke
FP2x	*P. sativum*	poke
FPD1	*P. sativum*	poke
FPD2	*P. sativum*	poke

* Isolate codes were chosen based on how the isolate was derived. PP = *L. venosus*, L = *L. culinarus*, FP = *P. sativum* and used as an identifier prior to proper identification. For example, PP1A1 represents the 1st *L. venosus* nodule plated on YEMA plate A, and 1st distinct single colony.

3.1.4 Identification of rhizobia

3.1.4.1 Taxonomic identification of isolates using 16S rRNA molecular analysis

Pure cultures of isolates (n = 17) obtained from nodules of *P. sativum* soil traps and University of Saskatchewan culture collections (n = 7), as well as field collected specimens (n=3) were chosen for 16S rRNA microbial identification. These cultures were streak-plated on YEMA from glycerol stock and then inverted and incubated at 23°C in the dark. DNA was extracted, and the 16S rRNA product was amplified by PCR using primers 27F/1492R by Insight Plant Health (3530 Millar Ave #210, Saskatoon, SK). The amplified PCR product was sent to Bio Basic Inc (20 Konrad Crescent, Markham, ON) for bidirectional Sanger sequencing. For each isolate, the results were assembled into a single contig and aligned using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) reference database. After alignment, isolates were taxonomically assigned to their closest known relative through this database.

A representative sample of bacteria for identification through Sanger sequencing of the 16S rRNA gene was determined by selecting isolates that had been evaluated in the dual plate bioassay (Section 4.1.1, p. 38) and growth pouch experiments (Section 4.2, p. 45) and exhibited additional characteristics of interest. It was deemed essential to include all three antagonistic isolates, each of which was isolated from *P. sativum* trap plants. Consequently, isolates were categorized into five groups: antagonistic (zones of antagonism); potential growth inhibitors; potential growth accelerators; likely non-antagonistic (no effect); and those used in the growth pouch experiment. Potential growth-inhibiting and accelerating effects were observed by measuring *A. euteiches* colony extension (Section 3.4.1, p. 26). Furthermore, isolates were screened for sequencing eligibility by confirming their growth on the bioassay media on which

they were evaluated. Approximately an equal number of isolates from each of the four soil sampling locations for *P. sativum* trap plants were included (excluding antagonistic isolates).

As Sanger sequencing of the 16S rRNA gene is costly, the representative sample aimed to minimize bias while identifying essential isolates, such as the three antagonistic ones or those used in the growth pouch experiment. This sub-sample allows for making inferences about the larger population.

3.2 Source of *Aphanomyces euteiches*

A pure culture of *A. euteiches* AE1 was obtained from the Crop Development Centre, University of Saskatchewan (courtesy of Dr. Sabine Banniza), and cultured according to Godebo et al. (2020). Briefly, potato dextrose agar (PDA) plates with a 5 mm diameter *A. euteiches* AE1 plug were incubated inverted for 5 d at 23°C in the dark. Subsequently, the cultures were either sub-cultured onto fresh PDA plates or stored as stock culture in vials containing sterile distilled H_2O at 4°C.

3.3 Evaluation of Media Compatibility for Putative Rhizobia and *Aphanomyces euteiches*

For a successful antagonism evaluation, the chosen medium must support the growth of all organisms involved. While *A. euteiches* has been assayed on PDA with bacteria isolated from soil (Godebo et al., 2020), rhizobia typically grow best on yeast extract mannitol agar (YEMA). The compatibility of putative rhizobia with PDA and *A. euteiches* with YEMA was uncertain.

To address this issue, putative rhizobia were first evaluated for their growth on PDA, and *A. euteiches* was assessed for its growth on YEMA. If neither putative rhizobia nor *A. euteiches*

grew well on each of the ideal media, two new mixed culture media were created: potato dextrose mannitol agar (PDMA) and potato dextrose yeast extract mannitol agar (PDYEMA).

All media recipes can be found in Appendix B. Additional information on the compatibility of isolates and *A. euteiches* with various culture media can be found in Appendix C.

3.4 Evaluation of Rhizobia and Soil Bacteria Isolates for Antagonistic Characteristics Towards *Aphanomyces euteiches* in Dual Plate Assays

3.4.1 Procedure for the dual plate bioassay

Fresh 72 h PDA, PDMA, PDYEMA, and YEMA plates were streak plated with pure putative rhizobia colonies from glycerol stock and agar slants and incubated at 23°C for 3 to 5 d until single colonies were observed. Two separate loopfuls of each culture were transferred with a sterile 1 µL inoculating loop to opposite sides of the plate, 1.5 cm from the edge. A 0.8 cm diameter core from a 5 d *A. euteiches* pure culture was centered on the plate. Control plates contained a single central *A. euteiches* core without bacteria. Four positive controls were included (i.e., plates with bacteria which formerly demonstrated antagonism towards *A. euteiches*) (Godebo et al., 2020).

A total of 72 isolates obtained through this study were subject to the bioassay (17 culture collection isolates, 42 soil trap isolates, and 13 isolates from field collected specimens). Bioassays were typically conducted in batches of 10 to 20 isolates. Through successive bioassays, different isolates were replicated to varying degrees on different culture media (Appendix D).

Potential growth-inhibiting and accelerating effects were observed by measuring *A. euteiches* colony extension with a mm ruler daily for 5 d throughout the dual plate bioassay on PDYEMA and YEMA.

3.4.2 Criteria for antagonism

Antagonistic characteristics between microorganisms can manifest in multiple ways. The most visually evident and commonly observed form is the presence of zones of antagonism. In a competitive environment, such as the one simulated in a dual plate bioassay, zones of antagonism may appear as halos surrounding a bacterium. To draw accurate conclusions (e.g., isolate y is not antagonistic towards *A. euteiches* on PDA), it is essential to first confirm that the bacteria can grow on the selected assay media. For more information on specific isolates and their compatibility with different culture media, refer to Appendix D.

Additionally, a non-antagonistic interaction referred to here as spreading behavior was observed after 7 d of incubation on YEMA (Fig. 3.3). Spreading behavior is defined here as a non-uniform bacterial growth outward from the original bacterial colony. Specifically, this behavior only occurred in the presence of *A. euteiches*. When bacteria were grown in absence of *A. euteiches*, the behavior did not occur (bacterial colonies expanded outwards uniformly).

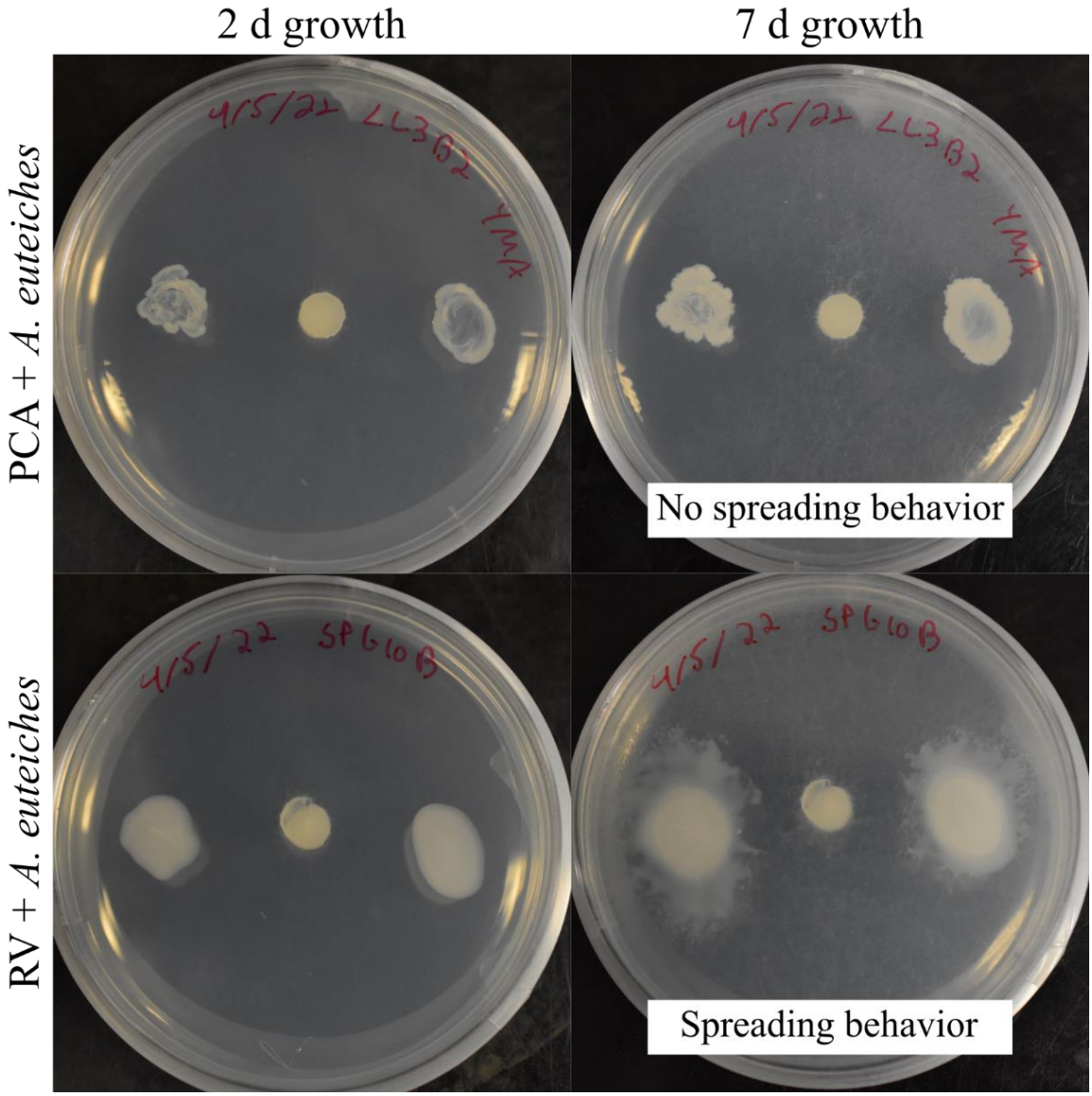

Figure 3.3 Visual description of observed spreading behavior. Isolate *Pseudomonas chlororaphis* subsp. *aurantiaca* strain LL3B2 (PCA) expands uniformly over time in the presence of *A. euteiches*. Conversely, isolate *R. leguminosarum* bv. *viciae* SPG10B (RV) does not expand uniformly, and instead spreads over the *A. euteiches* colony. This behavior only occurs in the presence of *A. euteiches*.

3.5 Modified Dual Plate Bioassay for the Evaluation of Nodule Associated Bacteria Antagonistic Synergies Towards *Aphanomyces euteiches*

Three isolates (*K. cowanii* LL3B1, *P. chlororaphis* subsp. *aurantiaca* LL3B2, and *Burkholderia* strain SU2B2) previously identified as antagonistic towards *A. euteiches* (Section 3.1.4, p. 23) and isolated from nodules of *P. sativum* trap plants, were assessed for their combined effects using a modified dual-plate assay on fresh PDYEMA. Additionally, *R. leguminosarum* bv. *trifolii* LPNI-2 (Germida, 1988) and *Agrobacterium* strain SU2A (previously shown to slow *A. euteiches* growth on YEMA and isolated from a *P. sativum* trap plant nodule) were included individually and in various combinations.

Each isolate was streak plated on PDYEMA and incubated for different time periods (Table 3.3). *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 was incubated for two different lengths of time to assess if there was any significant difference due to the incubation period (designated as LPNI-2A and LPNI-2B).

Table 3.3 Isolates of potential biocontrol agents included in the modified dual plate bioassay to evaluate potential synergistic interactions.

Isolate Code*	Closest Identification†	Incubation Period (days)	Description
LL3B1	*Kosakonia cowanii*	3	Antagonistic towards *A. euteiches*, cohabitant of LL3B2 nodule
LL3B2	*Pseudomonas chlororaphis* subsp. *aurantiaca*	3	Antagonistic towards *A. euteiches*, cohabitant of LL3B1 nodule
SU2B2	*Burkholderia* sp.	6	Antagonistic towards *A. euteiches*,
SU2A	*Agrobacterium* sp.	6	Slowed colony growth of *A. euteiches*
LPNI-2A	*Rhizobium leguminosarum* bv. *trifolii*	6	Previously demonstrated nodulation of *P. sativum* (de Freitas et al., 1993)
LPNI-2B	*Rhizobium leguminosarum* bv. *trifolii*	29	

* Isolate codes were used as an identifier prior to proper identification.

† Closest identification was determined from the closest match with >97% similarity when sequences were queried in the NCBI BLAST database.

Prior to the bioassay, two pure *A. euteiches* cultures were incubated on PDYEMA for 8 d. Cores were then transferred to the center of fresh PDYEMA plates in a biosafety cabinet using a sterile corer and sterile forceps. A loopful of each strain was transferred to opposite edges near the plate periphery using 1 µl sterile inoculating loops. For plates requiring a combination of strains, another loopful was placed in the same locations near the plate periphery. The order of strain transfer was random.

Each combination was replicated on three plates and incubated inverted at 23°C for 5 d, after which they were photographed. The primary objective was to examine the appearance of any zones of antagonism or other antagonistic interactions towards *A. euteiches*.

3.6 Gnotobiotic Evaluation of Select Nodule Isolates (Growth Pouch Experiments)

3.6.1 Experimental design

Two growth pouch experiments were conducted between May 2022 and August 2022 to determine if isolates could nodulate *P. sativum* as a liquid inoculant. At the time of the first two experiments, only the CIAT 632 isolate, identified as *Rhizobium leguminosarum* bv. *phaseoli* through FAME, had been taxonomically identified. The growth pouch experiments were performed using a modified method from Somasegaran and Hoben (1994). Specifically, the modifications included a revised nutrient solution recipe (Broughton & Dilworth, 1971), sterile techniques, and watering volumes.

The first experiment consisted of five treatments (*K. cowanii* LL3B1, *P. chlororaphis* subsp. *aurantiaca* LL3B2, *Burkholderia* strain SU2B2, and *Rhizobium* sp. CIAT 632) and an uninoculated negative control, which were treated every second day with 30 mL of sterile 0.05% KNO_3. In this experiment, many seeds failed to emerge, which was attributed to the lack of pre-germination in the growth pouch setting. Consequently, the results from this experiment were excluded from statistical analysis.

The second experiment included seven treatments (*K. cowanii* LL3B1, *P. chlororaphis* subsp. *aurantiaca* LL3B2, *Burkholderia* strain SU2B2, *Agrobacterium* strain SU2A, *Rhizobium leguminosarum* LL8B, and *Rhizobium leguminosarum* bv. *viciae* SPG10B) and a negative control treated every second day with 20 mL of 0.05% KNO_3. Pre-germination was used in this experiment, resolving the inability of seeds to reach the initial leaf developmental stage encountered in the first experiment.

In the first two experiments, each treatment was replicated using three growth pouches, with each pouch containing three surface-sterilized, and in the case of the second experiment pre-

germinated, cv. CDC Meadow *P. sativum* seeds. The uninoculated negative control was also replicated. The growth pouches were arranged in a completely randomized design. A third growth pouch experiment was also conducted in June 2023, which is further described in section 3.6.6 (p. 34-35).

3.6.2 Seed surface-sterilization and pre-germination

Seed surface-sterilization followed the procedure outlined in Vincent (1970). Briefly, a batch of 50 pea seeds was soaked in 50 mL of 65% v/v ethanol for 3 min in a 200 mL beaker, covered with a sterile Petri dish, and swirled gently. The ethanol was then drained, and the batch was transferred to a covered 200 mL beaker containing 50 mL of 1.2% v/v sodium hypochlorite for 5 min, swirling gently. After draining the sodium hypochlorite, the seeds were transferred to one of a series of 10 sterile Petri dishes pre-filled with enough sterile water to cover the bottom.

The seeds were swirled gently in each Petri dish, with the water being carefully drained before transferring the batch to another sterile dish. This process was repeated ten times. From the final wash dish, a small amount of water, approximately equal to the volume needed to cover the surface of the dish, was poured onto two separate, fresh YEMA dishes. These dishes were incubated for 7 d at 23°C. If no growth occurred on either replicate, the wash was considered sterile for that batch.

The seeds were then transferred to a folded paper towel, moistened with deionized water, and placed in a new clear plastic bag. Next, the bag was stored in a dark cupboard at room temperature and re-dampened after 2 d. After 4 d, the seeds were checked for germination. Both batches showed complete germination, as all seeds had formed a short radicle. The entire surface-sterilization procedure was repeated with fresh ethanol and sodium hypochlorite solutions, and new sterile Petri dishes for each batch of 50 seeds.

3.6.3 Determination of inoculant concentration

An uninoculated sterile Erlenmeyer flask with 25 mL of sterile yeast extract mannitol broth (YEMB) was incubated alongside the inoculants on a rotary shaker at 240 rpm for 3 d. All inoculants appeared turbid, while the negative control remained clear. To determine the colony forming units (CFU) of each inoculant, 0.1 mL aliquots from both 10^{-5} and 10^{-6} serial dilutions of each inoculant were spread-plated on three replicates each of YEMA and incubated for up to 7 d. The negative controls exhibited no growth after 7 d. When plates displayed 30 to 300 CFUs, the inoculant concentration was calculated.

3.6.4 Application of inoculants on surface-sterilized seeds

Sterile growth pouches were prepared by autoclaving on L-30 cycle (121°C for 30 min). Growth pouches were then prepared in a biosafety cabinet pre-treated with 15 min of UV exposure and wiped with 70% ethanol. Specifically, pouches were labelled and placed in growth racks in randomized positions, spaced approximately 4 cm apart. Three surface-sterilized and pre-germinated seeds were then transferred to the growth pouch wick using sterile forceps where they were spaced evenly with the radicle oriented downwards (Fig. 3.4).

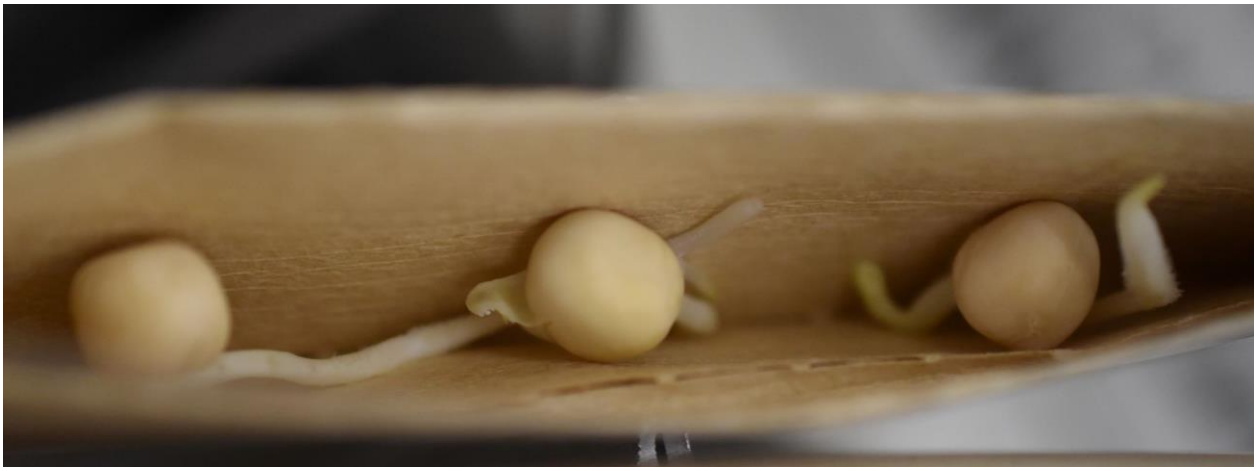

Figure 3.4 A sterile growth pouch containing evenly spaced pre-germinated surface-sterilized seeds.

For each pouch, 1.0 mL from each inoculant was pipetted directly onto the seed radicles of the respective pre-labelled pouch and replicate. Negative controls received 1.0 mL of sterile YMB instead. Care was taken to avoid any compromising position with the pipette that could result in cross-contamination. A new 1.0 mL sterile pipette tip was used for each pouch.

3.6.5 Growth conditions and plant watering

The plants were grown in a growth chamber set to a 16 h day and 8 h night cycle, with day temperatures at 23°C and night temperatures at 21°C. Light intensity ranged from 300 to 390 $umol \cdot m^{-2} \cdot s^{-1}$ and light bulbs were Phillips T-5 Fluorescence bulb #835. Before starting this experiment, the area used within the growth chamber was lightly rinsed with tap water to remove dirt and prevent thrips. Once in the growth chamber, the pouches were given 30 mL of sterile nutrient solution buffered to a pH of 6.6 to 6.8 (Recipe - Appendix B) and were incubated with an aluminum foil covering for 24 h.

Inoculated plants were watered once a day alternating between 30 mL sterile nutrient solution and D-H_2O. Negative controls received 0.05% KNO_3 buffered to 6.6-6.8 pH instead of D-H_2O. Solutions were pipetted slowly onto the wick of each pouch with a 10 mL pipette. Care was taken to avoid splash and all direct contact with any plant or pouch, although occasionally a splash would occur if the liquid encountered a leaf or stem near the wick.

For easier pipetting, solutions were first poured into a 2 L pitcher. Solutions were prepared in small batches (enough to last 2 d) to avoid prolonged sitting and potential growth of contaminants within the solutions. If the pipette did not encounter any splash or direct contact by mistake throughout a watering period, it was reused the following days. If any mistake occurred, the pipette was flushed with 70% ethanol and D-H_2O before proceeding to water nearby pouches.

After watering, the pitcher was emptied and remained in the growth chamber for re-use the following day.

After several weeks, plants became crowded with the 4 cm spacing on growth racks. Tendrils would occasionally intertwine. To observe roots, pouches would have to be picked up and tendrils manually unwoven by hand. After 5 weeks plants were removed from their pouches and examined for a final nodule count and root rot disease severity was analyzed and scored on a 1 to 5 scale following the procedure described by Wakelin et al. (2002). Several plants had nodules hidden underneath the growth pouch wick where that were not visible through the clear plastic growth pouch.

3.6.6 The third growth pouch experiment

In a third growth pouch experiment, plants were grown under the same conditions as the second growth pouch experiment and given the same sterile nutrient solution and water quantities. The experimental design was modified to be a completely randomized blind factorial design. The purpose of this experiment was to repeat the outcome of the second growth pouch experiment and evaluate interactions between potential biocontrol inoculants. Each unique treatment combination and control was replicated five times.

There were five treatments (*K. cowanii* LL3B1, *P. chlororaphis* subsp. *aurantiaca* LL3B2, *Agrobacterium* strain SU2A, *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B) as a single combined treatment containing up to a maximum of four isolates. In this experiment, there were two uninoculated control treatments. The first control received 0.5 mL of sterile YEMB, while the second control was given 30 mL of sterile 0.05% KNO_3 at 0 d and 15 d in addition to the sterile YEMB. To mitigate the risk of contamination

all treatments were watered with sterile distilled H_2O, and growth pouches were spaced at larger distances.

After 30 d plants were harvested and root nodules were counted. From ten root systems which scored the highest disease severity, a <1 cm long root sample was excised and sent to the Crop Development Centre, University of Saskatchewan to determine the identity of the causal root rot disease pathogen (courtesy of Dr. Sabine Banniza and Nimmlash Sivachandra Kumar). Briefly, roots were cut into small pieces and surface-sterilized with 10% sodium hypochlorite for 2 min followed by rinsing with sterile distilled water twice. Roots were dried on a paper towel before plating onto PDA. Plates were kept under fluorescent light for 24 hours then fungal species were identified based on the colony and spore morphology.

In addition, for each growth pouch, the respective roots were separated and placed in 1 L jars where they were subject to an acetylene reduction assay (ARA) to measure relative N fixation. Briefly, with a syringe 100 mL of air was expunged and then 100 mL of grade 2.6 atomic absorption C_2H_2 (99.6% pure) was injected into the jar and incubated for 1 h. Subsequently, a 20 mL sample was transferred to a 2 mL vacutainer. The gas samples were analyzed by the Gas Chromatography Lab, University of Saskatchewan for C_2H_4 using a Scion 456-GC and CombiPAL autosampler (CTC Analytics AG, Switzerland; purchased in 2015) and CompassCDS 4.0 software. Resulting ethylene production was calculated by first subtracting blank C_2H_4 values, which consisted of a 1 L jar subjected to the same ARA procedure, except without a root system. Following the ARA, roots and shoots were air dried for 3 d at 40˚C and was weighed using a pre-calibrated mg scale to determine dry biomass.

3.6.7 Statistical analysis

All statistical analyses were performed using R statistical analysis software (version 4.2.3). Acetylene reduction, nodule, and biomass results were analyzed using a one-way ANOVA test. It was confirmed that homogeneity of variance was equal between all groups by running a Bartlett's homogeneity of variance test. Residuals were tested to be normal with based on the Shapiro-Wilk test for normality. Means separations were determined with a Tukey HSD test.

The disease severity scores were analyzed using the non-parametric Kruskal-Wallis test to determine if there were any significant differences among the treatments and control. Following this test, post-hoc pairwise comparisons were conducted using Dunn's test with a Bonferroni correction for multiple comparisons.

4 RESULTS

4.1 Laboratory Studies

4.1.1 Dual plate bioassay

For ease of reading, a reference list of isolates and corresponding abbreviations frequently used in figures throughout the results section is provided (Table 4.1)

Table 4.1 Reference table of bacterial isolates and corresponding abbreviations used in figures throughout the results section.

Isolate	Abbreviation
Burkholderia strain SU2B2	BH
Kosakonia cowanii LL3B1	KC
Pseudomonas chlororaphis subsp. *aurantiaca* LL3B2	PCA
Agrobacterium strain *SU2A*	AB
Rhizobium leguminosarum bv. *trifolii* LPNI-2	RT
Rhizobium leguminosarum bv. *viciae* SPG10B	RV
Rhizobium leguminosarum LL8B	RL
Rhizobium tropici 9039	RT9
Rhizobium tropici CIAT899	RTC
Rhizobium leguminosarum bv. *phaseoli* CIAT 7100	RLP
Rhizobium sophorae LL3A	RS
Rhizobium strain SU3A	RSU

Only three of 72 isolates (*K. cowanii* LL3B1, *P. chlororaphis* subsp. *aurantiaca* LL3B2, and *Burkholderia* strain SU2B2) demonstrated *in vitro* antagonism towards *A. euteiches* (Table 4.2). The relative strength of antagonism is contrasted with four antagonistic isolates provided by Godebo et al. (2020), which were isolated from soil samples in Saskatchewan. Furthermore, *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 were both isolated from the same *P. sativum* trap plant root nodule. Of the 20 isolates identified as belonging to the family Rhizobiaceae and subject to the dual plate assay with *A. euteiches*, none displayed any zones of antagonism (Table 4.2).

Table 4.2 Dual-plate bioassay results of identified soil and nodule associated bacteria interacting with an *A. euteiches* colony on either PDA, PDMA, PDYEMA, or YEMA media after a 5 d growth period.

Isolate Code*	Source	Closest Identification§	Zones of Antagonism¶
K-Be-H_3	soil (Godebo et al., 2020)	*Lysobacter gummosus*	+++++
K-H_f-H_2	soil (Godebo et al., 2020)	*Lysobacter capsici*	+++++
SU2B2	*P. sativum* trap plant root nodule	*Burkholderia* sp.	+++
LL3B2	*P. sativum* trap plant root nodule	*Pseudomonas chlororaphis* subsp. *aurantiaca*	++
PCB2-6	soil (Godebo et al., 2020)	*Bacillus cereus*	++
K-CB_2	soil (Godebo et al., 2020)	*Bacillus cereus*	+
LL3B1	*P. sativum* trap plant root nodule	*Kosakonia cowanii*	-†
SU2A	*P. sativum* trap plant root nodule	*Agrobacterium* sp.	-
SU9B	*P. sativum* trap plant root nodule	*Bacillus magaterium*	-
SPG5B	*P. sativum* trap plant root nodule	*Rhizobium hidalgonense*	-
MJ7A	*P. sativum* trap plant root nodule	*Rhizobium indicum*	-
LL8B	*P. sativum* trap plant root nodule	*Rhizobium leguminosarum*	-
SPG6B	*P. sativum* trap plant root nodule	*Rhizobium leguminosarum*	-
LL10B	*P. sativum* trap plant root nodule	*Rhizobium leguminosarum* bv. *trifolii*	-
SPG10B	*P. sativum* trap plant root nodule	*Rhizobium leguminosarum* bv. *viciae*	-
LL3A	*P. sativum* trap plant root nodule	*Rhizobium sophorae*	-
MJ6A	*P. sativum* trap plant root nodule	*Rhizobium sophorae*	-
LL5A	*P. sativum* trap plant root nodule	*Rhizobium* sp.	-
SPG1B	*P. sativum* trap plant root nodule	*Rhizobium* sp.	-
SU3A	*P. sativum* trap plant root nodule	*Rhizobium* sp.	-
SU6A	*P. sativum* trap plant root nodule	*Rhizobium* sp.	-
CIAT7230	nodule bacteria, culture collection	*Rhizobium etli* bv. *phaseoli*	-
CIAT7100	nodule bacteria, culture collection	*Rhizobium leguminosarum* bv. *phaseoli*	-
CIAT7136	nodule bacteria, culture collection	*Rhizobium leguminosarum* bv. *phaseoli*	-
LPNI-2	nodule bacteria, (Germida, 1988)	*Rhizobium leguminosarum* bv. *trifolii*	-
CIAT899	nodule bacteria, culture collection	*Rhizobium tropici*	-
9039	nodule bacteria, culture collection	*Rhizobium tropici*	-
FP2	*P. sativum* root nodule	*Rhizobium leguminosarum* bv. *viciae*	-
PP3	*L. venosus* root nodule	*Bacillus cereus*	-
PP2C4	*L. venosus* root nodule	*Rhizobium sophorae*	-

* Isolate codes were used as an identifier prior to proper identification.

† Although no zone of antagonism was observed, swarming behavior resulted in strong antagonistic behavior towards *A. euteiches* (refer to Figure 4.2 for visual examples).

§ Closest identification was determined from the closest match with >97% similarity when sequences were queried in the NCBI BLAST database.

¶ A plus sign (+) indicates the relative size of the zone of antagonism with (+++++) being the largest and (+) being the smallest while remaining visually evident. A (-) indicates that no zones of antagonism were observed on the media used within the assay.

4.1.2 Non-Rhizobiaceae family nodule associated bacteria demonstrated *in vitro* antagonism towards *Aphanomyces euteiches*

Pseudomonas chlororaphis subsp. *aurantiaca* LL3B2 and *Burkholderia* strain SU2B2 displayed similar zones of antagonism across replicated PDA, PDMA, and PDYEMA culture media (Fig. 4.1). Isolate *K. cowanii* LL3B1 was, in several instances, able to completely stop *A. euteiches* growth when monitored for 30 d (Fig. 4.2). All three of these antagonistic isolates did not consistently display the same antagonism when grown on YEMA, despite YEMA supporting good growth of the bacteria. Consequently, these results indicate that media selection can alter dual-plate bioassay outcomes.

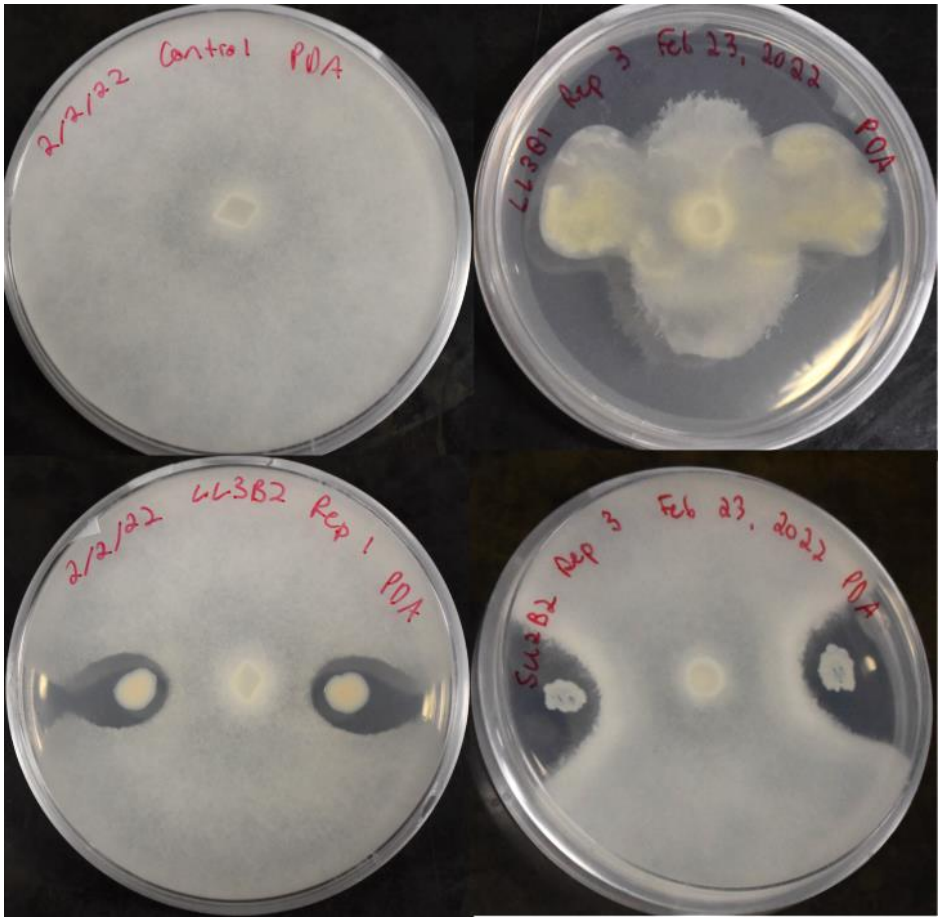

Figure 4.1 Dual-plate bioassay results for the three antagonistic isolates after incubating for 5 d on PDA. (Top left – control consisting of only *A. euteiches*, top right – *Kosakonia cowanii* LL3B1 (KC), bottom left *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), bottom right *Burkholderia* strain SU2B2 (BH). PCA and BH both showed zones of antagonism (clearing zones around the bacteria) whereas KC displayed a different type of antagonism.

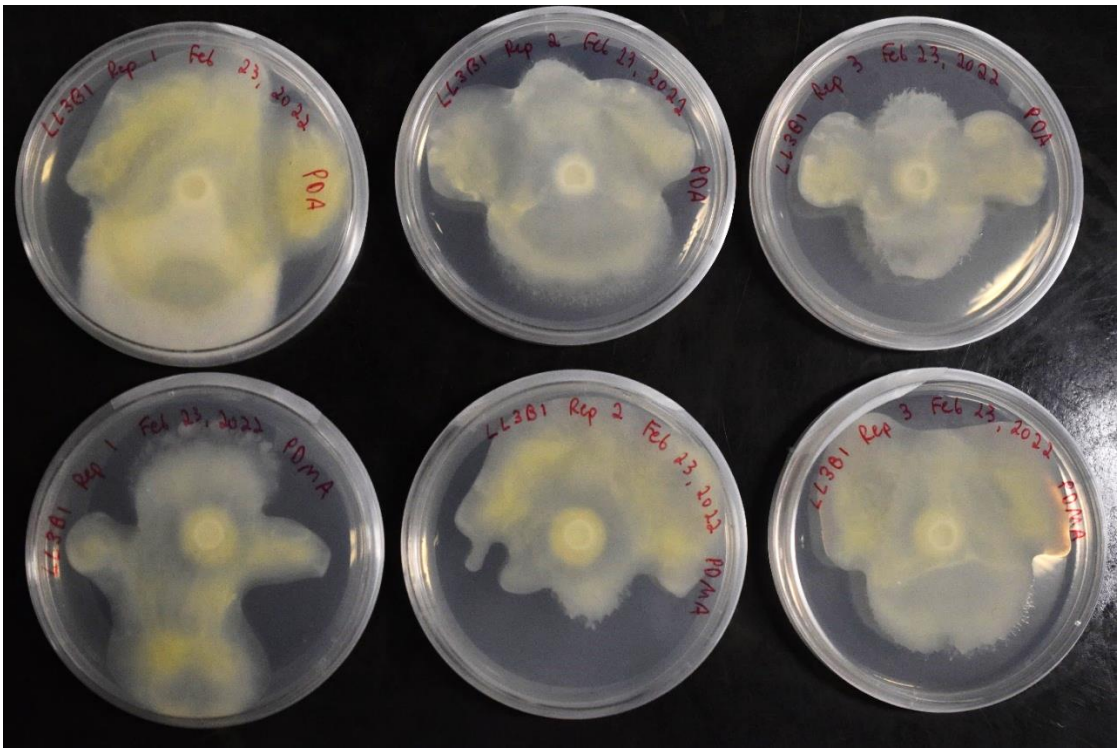

Figure 4.2 A swarming interaction was unique to *Kosakonia cowanii* LL3B1, where the bacteria would grow towards *A. euteiches* and in several replicates was completely able to stop the growth of *A. euteiches* for >30 d.

4.1.3 Rhizobia demonstrate spreading behavior in the presence of *Aphanomyces euteiches*

For cultures subject to the dual-plate bioassay on YEMA all non-antagonistic cultures demonstrated a spreading behavior 7 d after inoculation (Table 4.3 and Fig. 4.3). *Burkholderia* strain SU2B2 demonstrated this behavior to a stronger degree than all other bacteria and showed similar culture morphology to the non-antagonistic bacteria. *Kosakonia cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 did not demonstrate the same type of spreading behavior outwards from colonies (Fig. 4.3).

Table 4.3 Number and type of bacterial isolates which demonstrated a spreading behavior over *A. euteiches* hyphae after 8 d incubation.

Bacteria Type	Culture Media	n*	Spreading Behavior (n) †
antagonistic nodule bacteria	YEMA	3	2
culture collection	YEMA	10	10
P. sativum trap plant isolates	YEMA	24	24

* n refers to the number of unique bacterial isolates examined

† number of unique bacterial isolates which demonstrated a spreading behavior after a 7 d incubation period when grown with *A. euteiches*

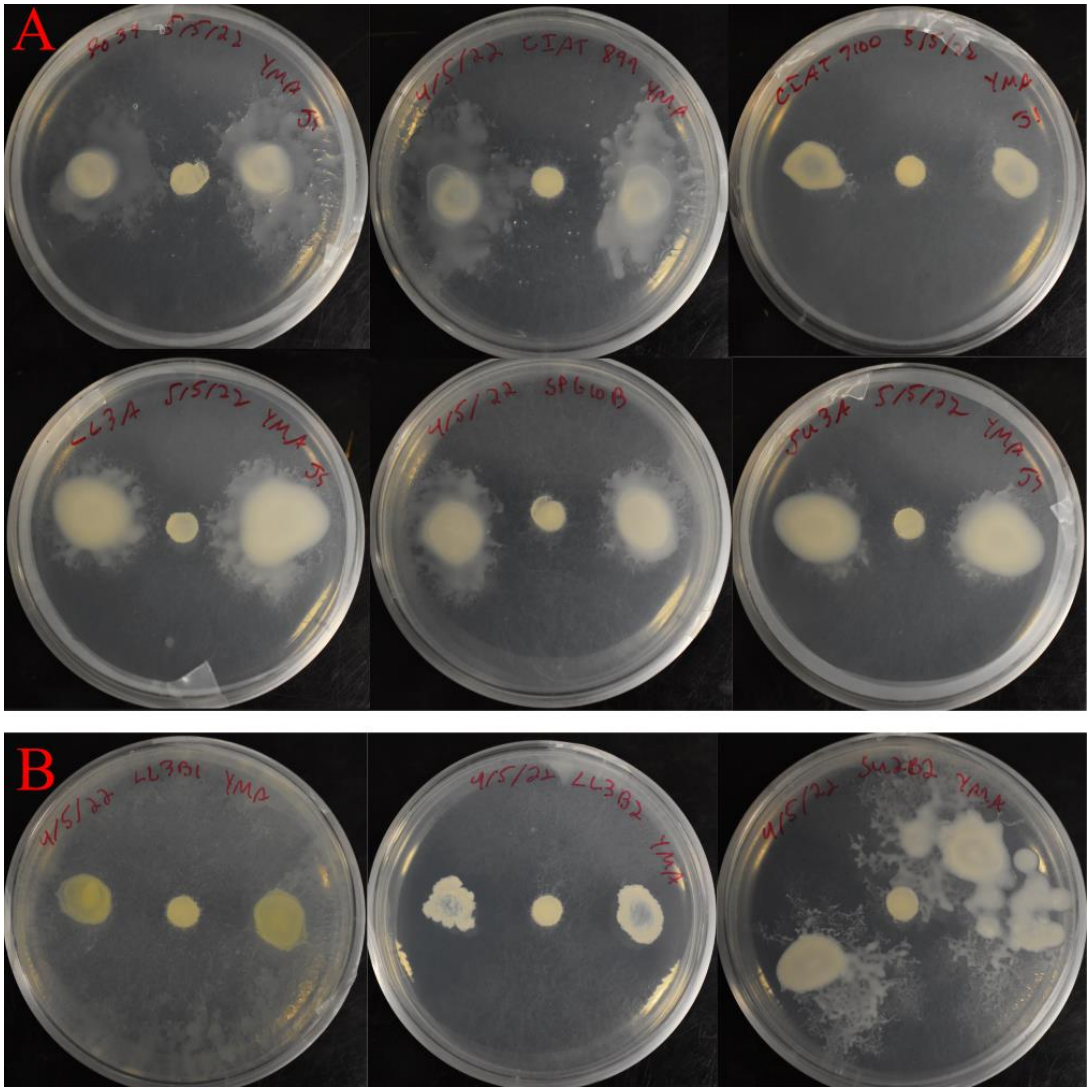

Figure 4.3 Spreading behavior of isolates over a central *A. euteiches* colony after 7 d of incubation. (A) Examples of non-antagonistic *Rhizobium* spp. isolates demonstrating this behavior, from left to right: Row 1 – (1) *Rhizobium tropici* 9039 (RT9), (2) *Rhizobium tropici* CIAT899 (RTC), (3) *Rhizobium leguminosarum* bv. *phaseoli* CIAT 7100 (RLP); Row 2 – (1) *Rhizobium sophorae* LL3A (RS), (2) *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV), (3) *Rhizobium* strain SU3A (RSU). (B) Antagonistic isolates after the same incubation period on YEMA, from left to right: (1) *Kosakonia cowanii* LL3B1 (KC), (2) *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), and (3) *Burkholderia* strain SU2B2 (BH).

All bacteria which were identified as belonging to the Rhizobiaceae family spread over *A. euteiches* to varying degrees.

4.1.4 Indications that Rhizobiaceae and non-Rhizobiaceae family bacteria are synergistically antagonistic towards *Aphanomyces euteiches*

Non-Rhizobiaceae family nodule associated bacteria which demonstrated *in vitro* antagonism towards *A. euteiches* appeared to have enhanced antagonism when grown with *R. leguminosarum* bv. *trifolii* LPNI-2 on PDYEMA culture medium. In *P. sativum*, *R. leguminosarum* bv. *trifolii* LPNI-2 is known to nodulate *P. sativum* and fix atmospheric N_2.

Nodule cohabitants LL3B1 and LL3B2 had more pronounced antagonism when applied in combination (Fig. 4.4). Furthermore, they were increasingly effective with the addition of the known nodule forming *R. leguminosarum* bv. *trifolii* LPNI-2 (Figs. 4.5 – 4.9) where one replicate was able to stop the growth of the *A. euteiches* colony for >6 months (Plate 5 in Fig. 4.6).

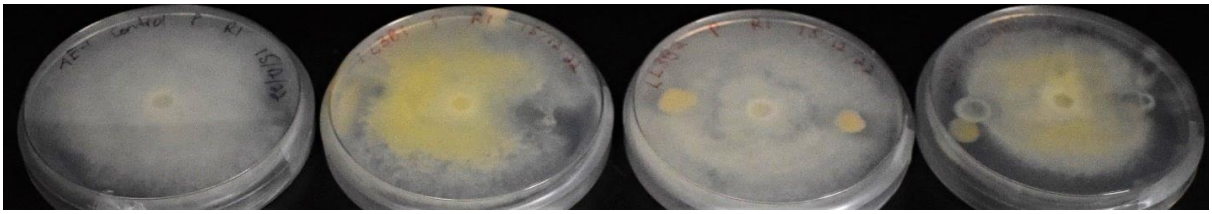

Figure 4.4 Dual plate bioassay synergistic outcomes for combined isolates against *A. euteiches*. Each PDYEMA plate consisted of a central *A. euteiches* colony and a combination of isolates as follows (from left to right): (1) Control, (2) *Kosakonia cowanii* LL3B1 (KC), (3) *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), (4) KC + PCA.

Figure 4.5 Dual plate bioassay outcomes for combined isolates against *A. euteiches*. Each PDYEMA plate consisted of a central *A. euteiches* colony and a combination of isolates as follows (from left to right): (1) Control, (2) *Kosakonia cowanii* LL3B1 (KC), (3) *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), (4) *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 (RT), (5) KC + PCA + RT.

Figure 4.6 Dual plate bioassay synergistic outcomes for combined isolates against *A. euteiches*. Each PDYEMA plate consisted of a central *A. euteiches* colony and a combination of isolates as follows (from left to right): (1) Control, (2) *Kosakonia cowanii* LL3B1 (KC), (3) *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), (4) *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 (RT), (5) KC + PCA + RT.

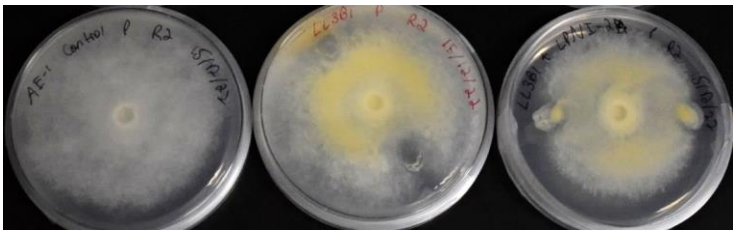

Figure 4.7 Dual plate bioassay synergistic outcomes for combined isolates against *A. euteiches*. Each PDYEMA plate consisted of a central *A. euteiches* colony and a combination of isolates as follows (from left to right): (1) Control, (2) *Kosakonia cowanii* LL3B1 (KC), (3) KC + *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 (RT).

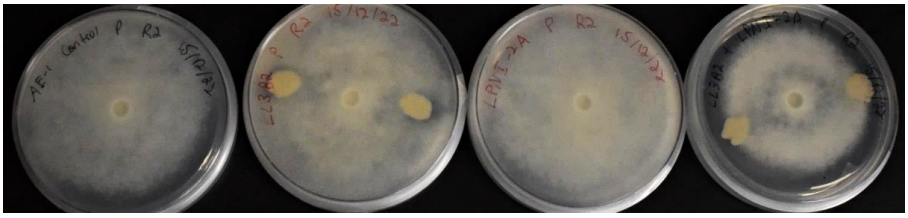

Figure 4.8 Dual plate bioassay synergistic outcomes for combined isolates against *A. euteiches*. Each PDYEMA plate consisted of a central *A. euteiches* colony and a combination of isolates as follows (from left to right): (1) Control, (2) *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), (3) *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 (RT), (4) PCA + RT.

The same effect was also observed when *Burkholderia* strain SU2B2 was included in the combination (Fig. 4.9).

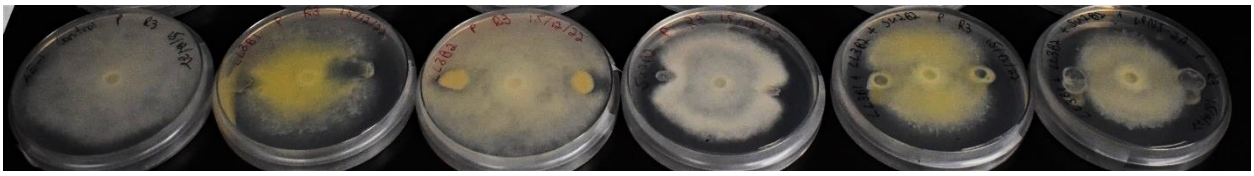

Figure 4.9 Dual plate bioassay synergistic outcomes for combined isolates against *A. euteiches*. Each PDYEMA plate consisted of a central *A. euteiches* colony and a combination of isolates as follows (from left to right): (1) Control, (2) *Kosakonia cowanii* LL3B1 (KC), (3) *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), (4) *Burkholderia* strain SU2B2 (BH), (5) KC + PCA + BH, (6) KC + PCA + BH + *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 (RT).

4.2 Growth Chamber Studies

4.2.1 Impact of inoculants on plant growth characteristics

For each isolate applied as a liquid seed inoculant on pregerminated seeds, all seeds proceeded to initial leaf development after 3 d.

Compared with the uninoculated control supplemented with 0.05% KNO_3 every other day, *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 both independently increased nodulation (*P*=0.10 and 0.27, respectively) in the second growth pouch experiment (Fig. 4.10). However, this outcome was not repeated in the third growth pouch experiment (Fig. 4.11). Despite following a standard seed surface-sterilization procedure, controls still formed nodules. Control nodulation may have been attributed to random contamination or other unknown factors. Additionally, *Burkholderia* strain SU2B2 and *R. leguminosarum* strain LL8B did not increase nodule counts (Appendix E, Fig. E.1, p. 89).

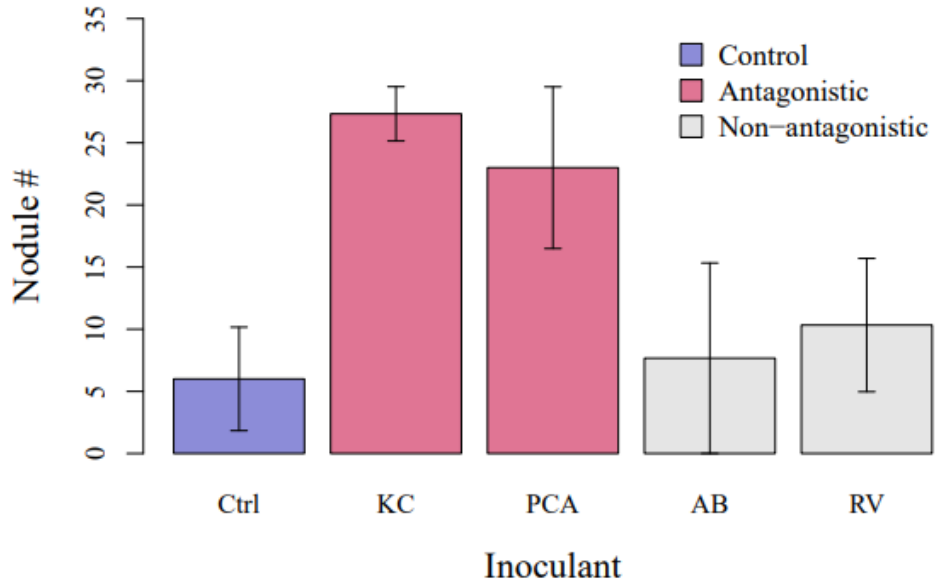

Figure 4.10 *Pisum sativum* nodule count outcome after 5 wks for inoculants *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), and *Rhizobium leguminosarum* bv. *viciae* (RV). Control was uninoculated and supplemented with 0.05% KNO_3 (Ctrl) every other day. Bars represent the mean of three replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Data is from the second growth pouch experiment. No significant differences detected ($p < 0.05$).

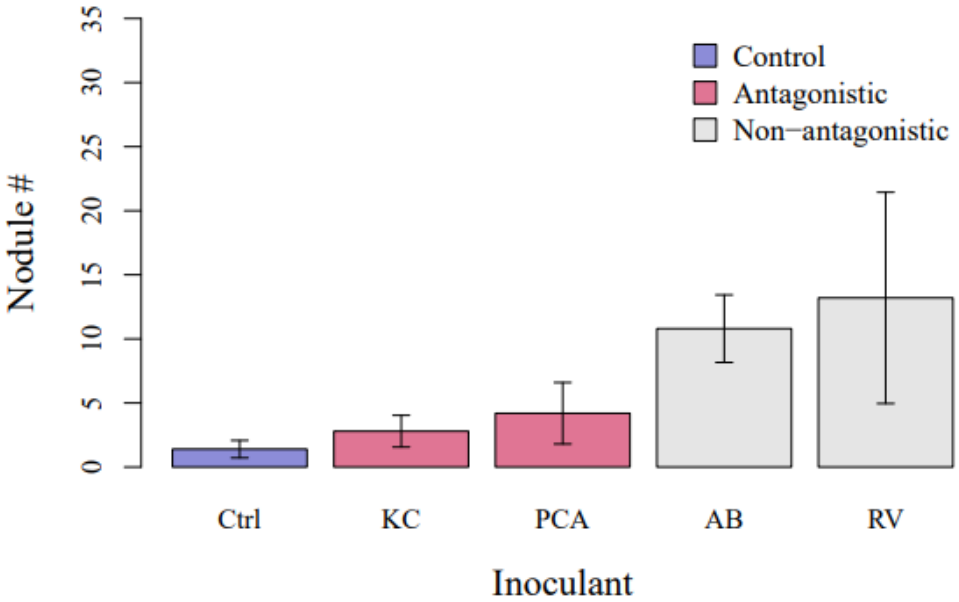

Figure 4.11 *Pisum sativum* nodule count outcome after 30 d for inoculants *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Control was uninoculated N-free (Ctrl). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Data is from the third growth pouch experiment. No significant differences were detected ($p < 0.05$).

In the third growth pouch experiment, the uninoculated control supplemented with 0.05% KNO_3^- (+N) at 0 and 15 d formed the most root nodules, and the uninoculated N-free control (-N) formed the least root nodules of all treatments (Fig. 4.12). Of the biological inoculant treatments, the known nodulators *Rhizobium leguminosarum* bv. *viciae* SPG10B and *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 independently formed the most root nodules. As a combined inoculant *K. cowanii* LL3B1 + *P. chlororaphis* subsp. *aurantiaca* LL3B2 + *Agrobacterium* strain SU2A + *Rhizobium leguminosarum* bv. *trifolii* LPNI-2 formed the most root nodules.

As independent inoculants, both *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 produced relatively few nodules (Fig. 4.12), contradicting the outcome observed in the second growth pouch experiment in which both inoculant treatments were associated with nodule production (Fig. 4.10).

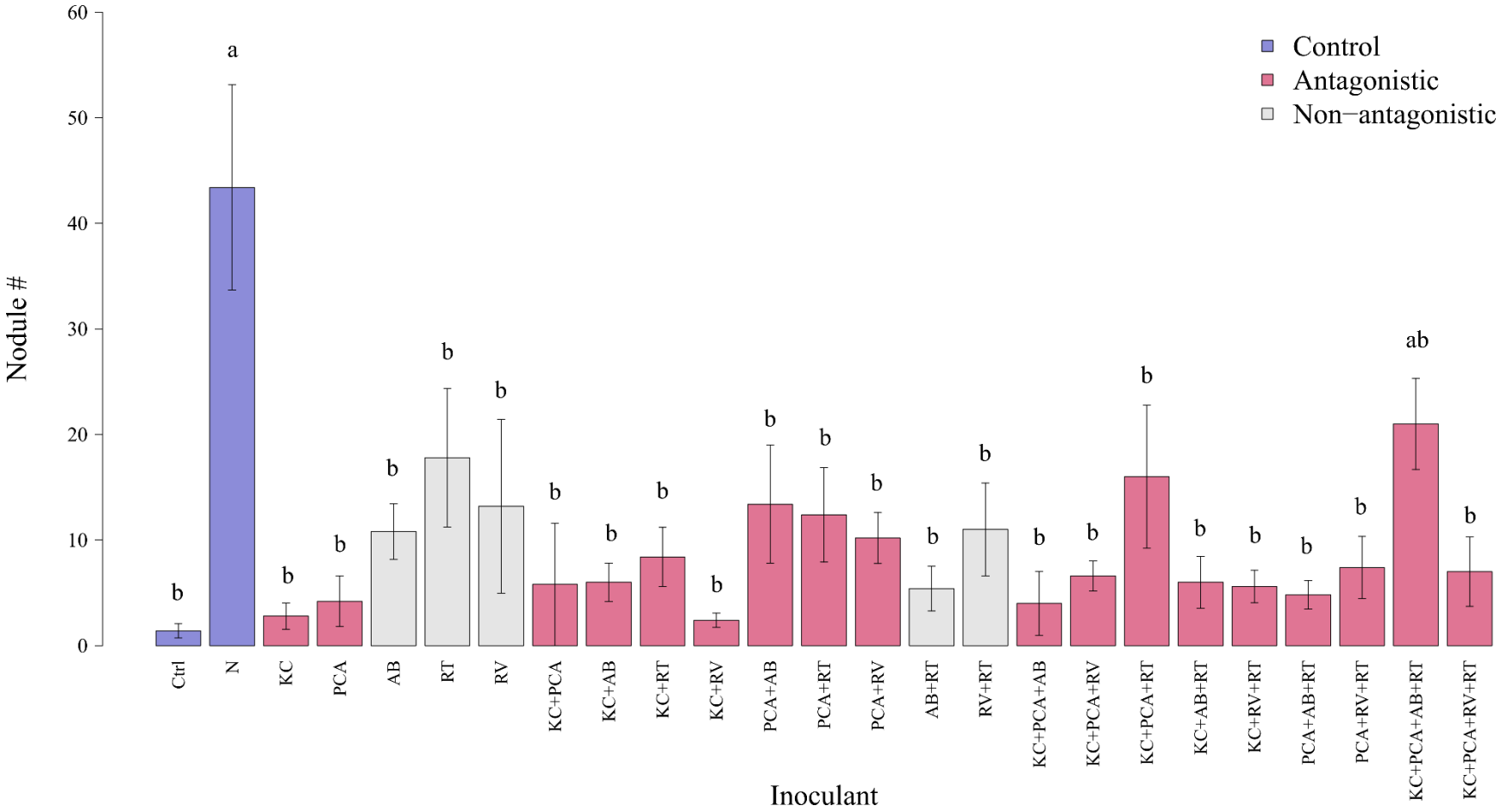

Figure 4.12 *Pisum sativum* nodule count after 30 d for each inoculant combination: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Combinations are represented by isolate abbreviations separated by a plus (+) sign. Controls were uninoculated N-free (Ctrl) and uninoculated supplemented with 0.05% KNO_3 (N). Error bars are standard error. Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Data is from the third growth pouch experiment. Letters represent significant differences ($p < 0.001$).

In terms of C_2H_4 production, the +N uninoculated control produced a similar quantity to some of the best performing combined inoculants, such as KC+PCA+AB+RT (Fig. 4.13). As expected, the N-free uninoculated control did not produce appreciable levels of C_2H_4, nor did the KC+PCA+AB combination. The addition of RT to the combined inoculant KC+PCA+AB nearly doubled the C_2H_4 production relative to RT alone, suggesting a positive interaction occurred between these isolates as combined inoculants.

Applied as single inoculants, all isolates induced production of C_2H_4 relative to the uninoculated control. *Kosakonia cowanii, P. chlororaphis* subsp. *aurantiaca*, and *Agrobacterium* strain SU2A independently induced C_2H_4 production, however, the combination of all three (KC+PCA+AB) resulted in less C_2H_4 production than the uninoculated control (Fig. 4.13).

Total, root, and shoot dry biomass varied between treatments in the third growth pouch experiment (Appendix E, Figs. E.4-6, p. 92-94).

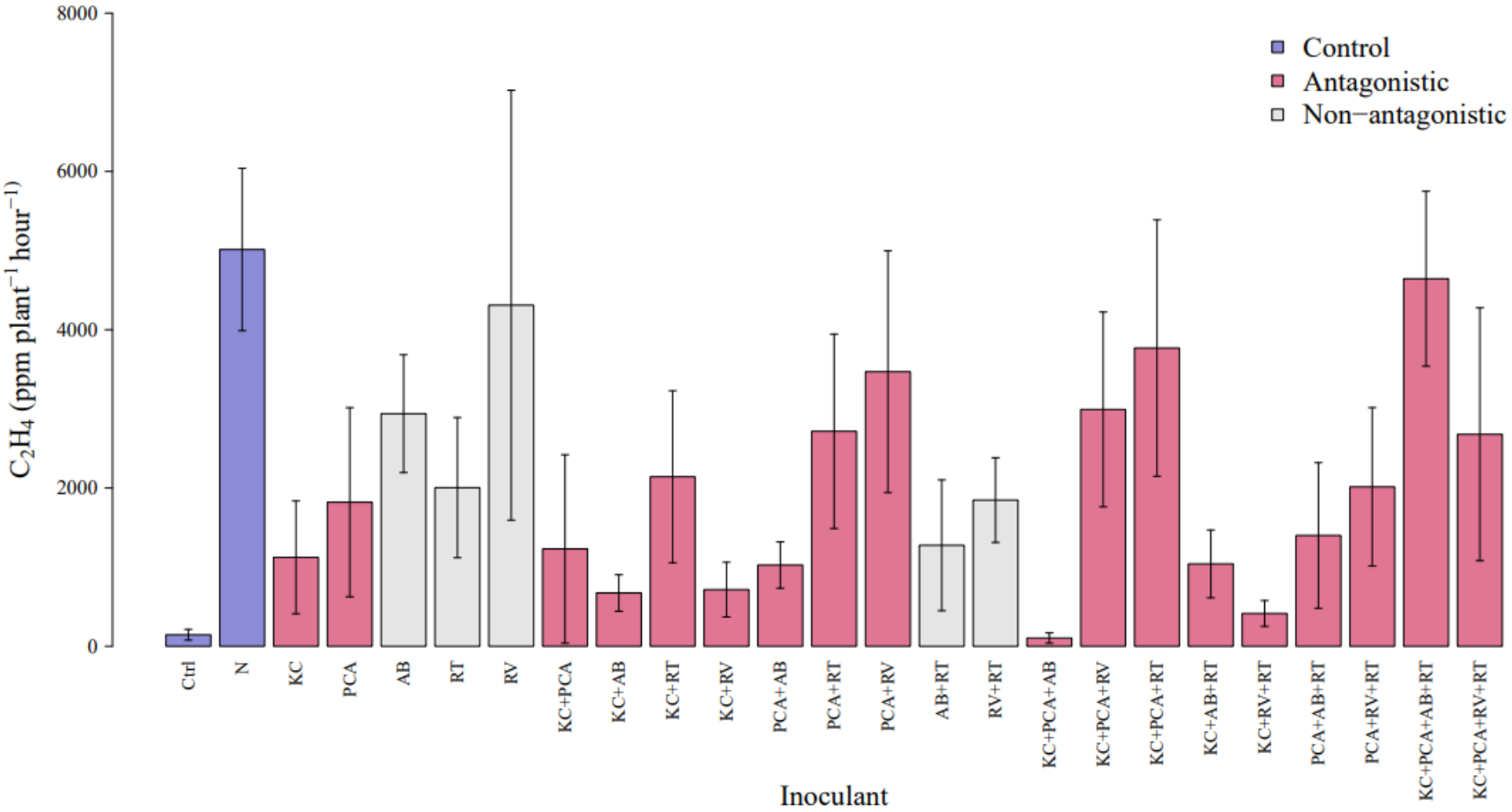

Figure 4.13 *Pisum sativum* nodule count after 30 d for each inoculant combination: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Combinations are represented by isolate abbreviations separated by a plus (+) sign. Controls were uninoculated N-free (Ctrl) and uninoculated supplemented with 0.05% KNO_3 (N). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* plants. Error bars are standard error. Data is from the third growth pouch experiment. No significant differences ($p < 0.05$) were detected.

4.2.2 Impact of inoculants on disease development

In the second and third growth chamber experiments, nearly half of all growth pouches developed root rot symptoms of varying severity over the 5 wk growing period. Although unintended, the randomized experimental design allowed for isolates to be scored based on their disease severity outcomes (Figs. 4.14 and 4.15).

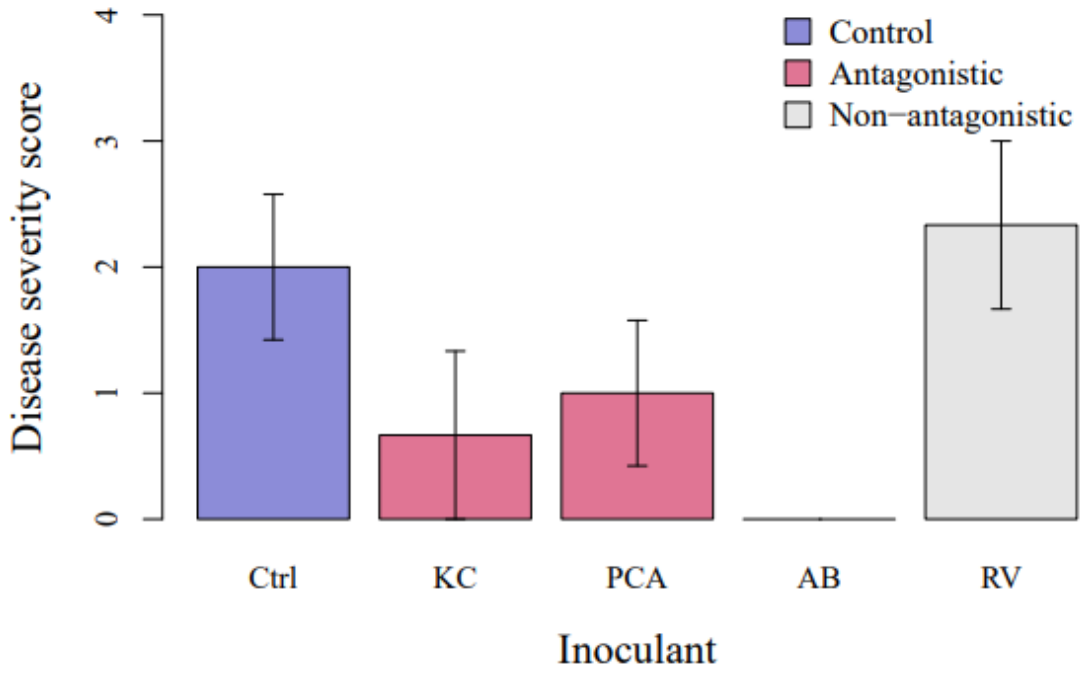

Figure 4.14 *Pisum sativum* root rot disease severity outcomes after 5 wks for each inoculant: *Kosakonia cowanii* LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), and *Rhizobium leguminosarum* bv. *viciae* (RV). Control was uninoculated and supplemented with 0.05% KNO_3 (Ctrl). Bars represent the mean of three replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Disease severity was scored following the procedure described by Wakelin et al. (2002). 0 = no symptoms; roots healthy and white. 1 = initial symptoms of root rot; discoloration, usually a light tan color, in sections. 2 = discoloration of most or all of the root system, usually still a tan color. 3 = advanced disease symptoms; extensive darkening and discoloration of the root system and extensive lesion formation. 4 = root entirely rotted/plant dead. Growth pouch positions were randomized. Data is from the second growth pouch experiment. No significant differences were detected ($p < 0.05$).

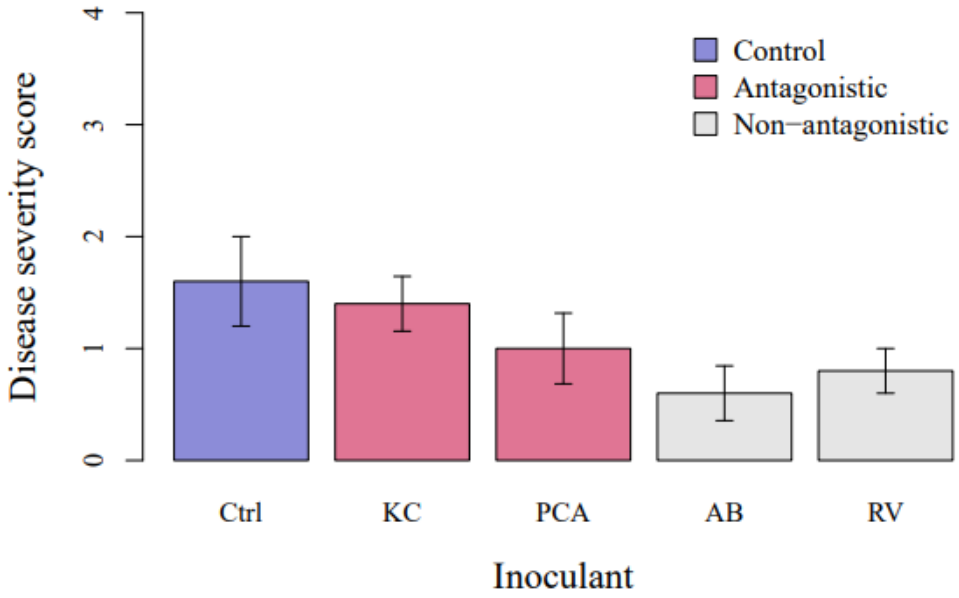

Figure 4.15 *Pisum sativum* root rot disease severity outcomes after 30 d for each inoculant: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), and *Rhizobium leguminosarum* bv. *viciae* (RV). Control was uninoculated N-free (Ctrl). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Disease severity was scored blind following the procedure described by Wakelin et al. (2002). 0 = no symptoms; roots healthy and white. 1 = initial symptoms of root rot; discoloration, usually a light tan color, in sections. 2 = discoloration of most or all of the root system, usually still a tan color. 3 = advanced disease symptoms; extensive darkening and discoloration of the root system and extensive lesion formation. 4 = root entirely rotted/plant dead. Growth pouch positions were randomized. Data is from the third growth pouch experiment. No significant differences were detected ($p < 0.05$).

No significant differences in disease severity were found between treatments ($P > 0.05$ for all pairwise comparisons). Additional disease severity score data for all treatments can be found in the Appendices (Appendix E, Figs. E.2 and E.3, p. 90-91).

Although the differences were not statistically significant, visual observations indicated that some treatments, such as *K. cowanii* LL3B1, *P. chlororaphis* subsp. *aurantiaca* LL3B2, and *Agrobacterium* strain SU2A, had relatively healthier root systems compared to the control. Additionally, their roots and nodules were visually less diseased than those belonging to non-antagonistic isolates *R. leguminosarum* strain LL8B and *R. leguminosarum* bv. *viciae* SPG10B. (Fig. 4.16).

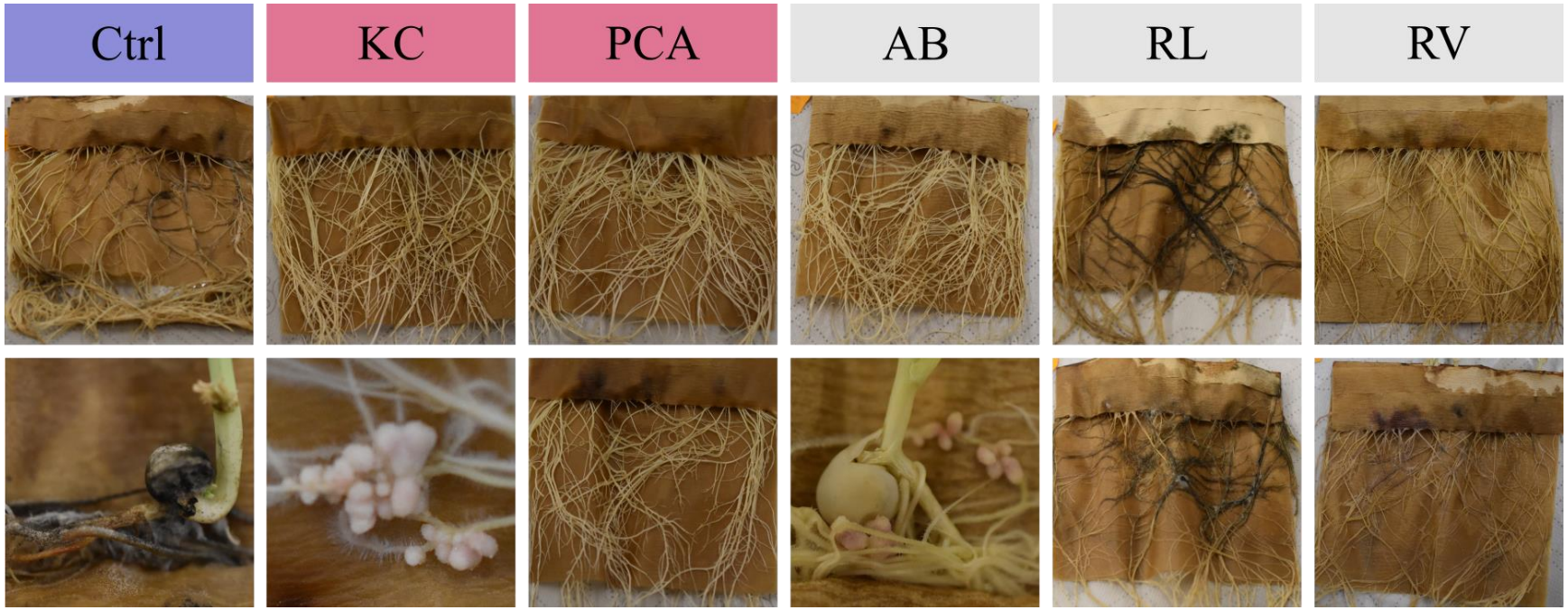

Figure 4.16 Visual root rot disease severity outcomes after 5 wks in *P. sativum* growth pouches for each inoculant: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), and *Rhizobium leguminosarum* bv. *viciae* (RV). Control was uninoculated and supplemented with 0.05% KNO_3 (Ctrl). The control, RL, and RV treatments had root systems with greater disease severity compared with those inoculated with antagonistic isolates KC and PCA, and non-antagonistic isolate AB. Root rot disease also caused varying nodule senescence. Data is from the second growth pouch experiment.

Root pathogens present were confirmed as *Alternaria*, *Fusarium redolens*, and *Fusarium solani* (Fig. 4.17). *Aphanomyces euteiches* was not detected in the diseased root samples.

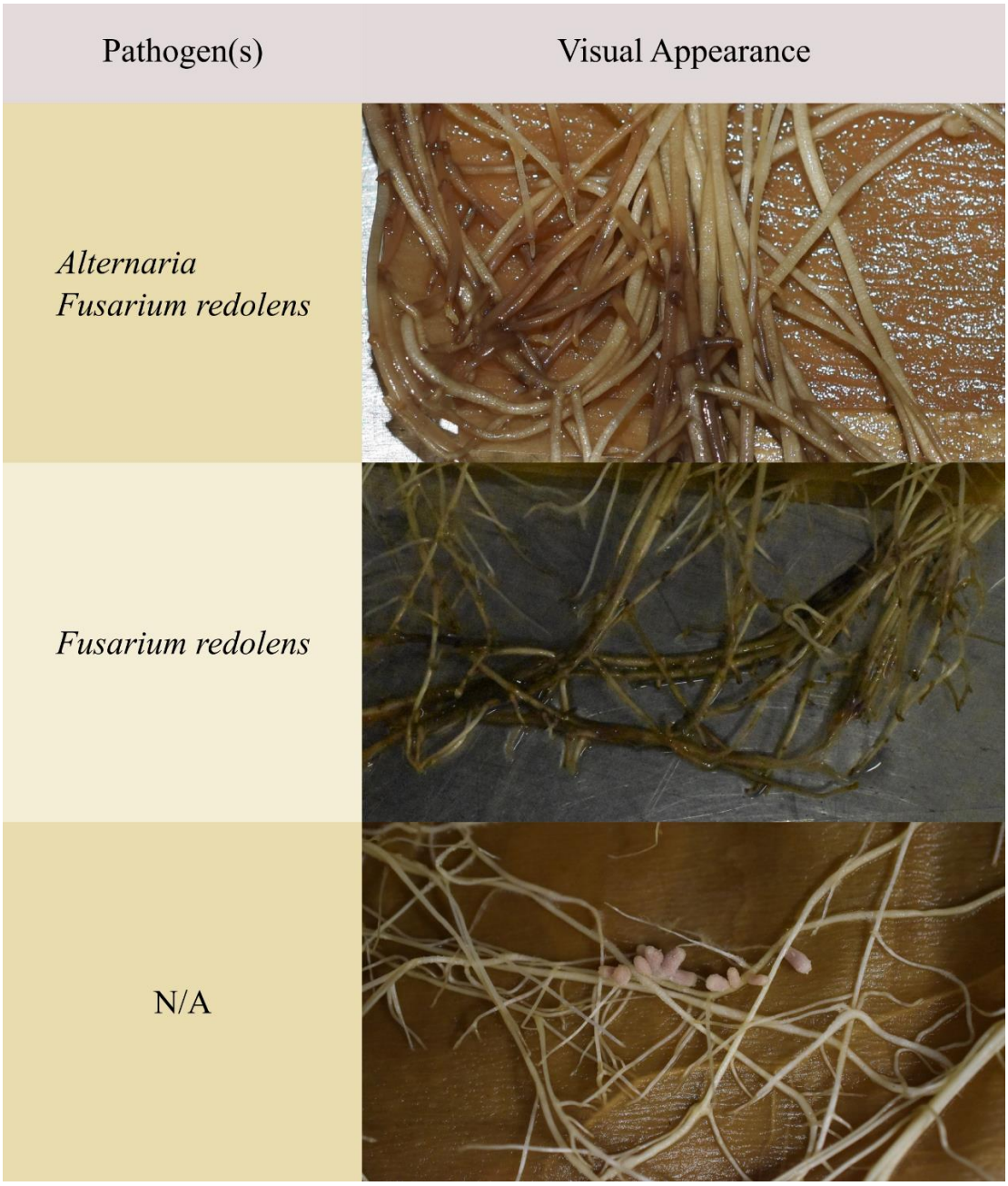

Figure 4.17 *Pisum sativum* roots infected by *Alternaria* and *Fusarium redolens*, alongside a non-infected root system. Host plants were grown in growth pouches for 30 d. Images from the third growth pouch experiment.

4.3 Diversity of Rhizobiaceae Family Bacteria Associated with *Pisum sativum* Nodules

Of the bacteria isolated from the nodules of *P. sativum* trap plants grown in field collected Saskatchewan soils, a total of 13 isolates were identified as belonging to the Rhizobiaceae family, representing seven distinct species (Fig. 4.18).

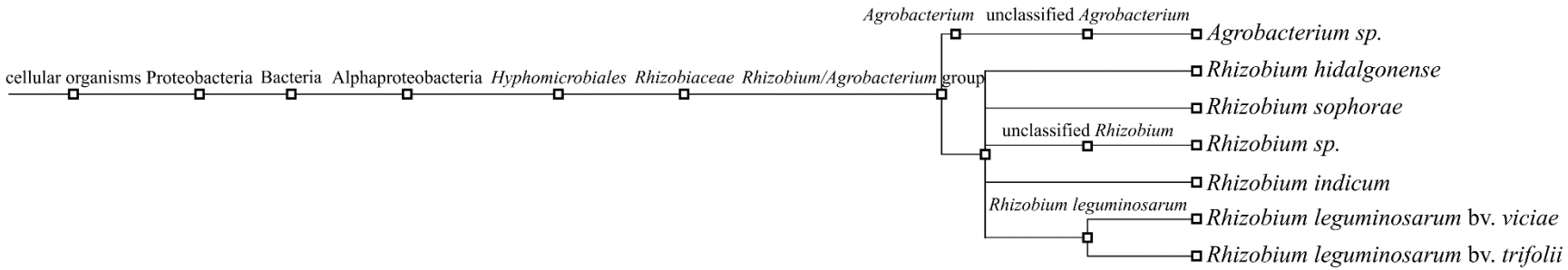

Figure 4.18 Phylogenetic tree of species identified as members of the Rhizobiaceae family based on 16S rRNA analysis. These species were isolated from the nodules of *P. sativum* trap plants grown in field collected Saskatchewan soils. The tree was generated with Interactive Tree of Life (iTOL) and the NCBI BLAST reference database.

5 DISCUSSION

5.1 *Rhizobium* spp. are Generally not Antagonistic Towards *Aphanomyces euteiches*

Results of this study suggest that *Rhizobium* spp. examined *in vitro* generally were not antagonistic towards *A. euteiches*. After a thorough literature review, there are very few papers which discuss *in vitro* antagonism by *Rhizobium* spp. towards *A. euteiches*. Perhaps the most relevant findings are that of Dileep Kumar et al. (2001). Here, three *R. leguminosarum* bv. *viceae* strains were evaluated on both YEMA and PDA for antagonism towards *A. euteiches*. Two of three strains showed significant antagonism on PDA, and one of three strains showed significant antagonism on YEMA. However, only very small zones of inhibition (0.5-0.6 cm) were apparent. The method used by Dileep Kumar et al. (2001) to assay *in vitro* antagonism was very similar to that used in this study. Similarly, Godebo et al. (2020) found 184 soil bacteria antagonistic towards *A. euteiches*, of which two were identified as *R. lemnae* isolates.

Aside from interactions directly with *A. euteiches*, more reports exist with regards to rhizobia and other phytopathogens comprising the Pea Root Rot Complex (PRRC). Bardin et al. (2004) found that *R. leguminosarum* bv. *viceae* can control *Pythium* damping-off of pea. However, only one of 18 *Rhizobium* spp. isolates tested, *R. leguminosarum* bv. *viceae* R5 inhibited the mycelial growth of *Pythium*. Contrasting with other fungal pathogens of the PRRC, *Pythium* is an oomycete. As such, it may be more representative when trying to draw conclusions relevant to *A. euteiches*.

Comparatively, Hemissi (2011) found that 24 of 42 *Rhizobium* spp. isolates demonstrated *in vitro* antagonism towards the fungal phytopathogen *Rhizoctonia solani*. These isolates were obtained from the nodules of 50 d old chickpea (*Cicer arietninum L.*) plants. Hemissi (2011) conducted a similar dual plate assay using PDA culture media; however, instead they applied 50

µL of a 10^8 cells mL^{-1} rhizobia suspension. This concentration is drastically higher than the 1 µL used in this study. Additionally, Hemissi (2011) also pre-incubated the rhizobia on PDA for 1 d before placing the *R. solani* colony at the centre of the plate.

This research has provided evidence that *Rhizobium* spp. isolated from *P. sativum* nodules generally do not demonstrate direct antagonism towards *A. euteiches*. Conversely, rhizobia could be further examined for their indirect antagonism towards *A. euteiches*. For instance, in Huang and Erickson (2007), three of four *R. leguminosarum* bv. *viceae* strains significantly reduced the incidence of damping-off caused by *Pythium* spp.. *Pythium* spp. is another oomycete pathogen comprising the PRRC in *P. sativum*. A key difference is that Huang and Erickson (2007) applied the *R. leguminosarum* bv. *viceae* strains as seed inoculants in fields naturally infected with *Pythium* spp. However, they did not evaluate the direct interaction between the oomycete-pathogen and rhizobia. Therefore, it is plausible that their results are from other benefits provided to *P. sativum* upon rhizobial inoculation as opposed to rhizobia being directly antagonistic towards *Pythium* spp..

5.2 Non-Rhizobial Nodule Endophytes as Biocontrol Agents

In this study, non-rhizobial nodule endophytes (NREs) demonstrated potential as feasible biocontrol agents for *A. euteiches*. These NREs may represent a group of relatively unexamined bacteria that naturally associate with legumes through nodule symbiosis. Accordingly, this may represent a reservoir of untapped applications in modern agriculture. In this group are the bacterial isolates *K. cowanii* LL3B1, *P. aurantiaca* LL3B2, and *Burkholderia* strain SU2B2, which demonstrate antagonistic behavior towards *A. euteiches*. Certain NREs can enhance protection against plant pathogens (Tokgöz et al., 2020). For instance, when co-inoculated with *Rhizobium* strain AAU B3, the NREs *Bacillus* strain AAU B6 and B12 increased antagonism against the root rot pathogen *Macrophomina phaseolina* in *Vigna radiata* (Preyanga et al., 2021). Similarly, the antagonistic behavior of *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 intensified in the presence of *R. leguminosarum* bv. *viciae* LPNI-2 (Figs. 4.5 to 4.8, p. 42-43). Numerous NREs have previously demonstrated various plant growth-promoting (PGP) traits, such as enhancing nodulation of *L. culinarus* and *Arachis hypogaea* (Preyanga et al., 2021; Debnath et al., 2023).

Moreover, co-inoculation of NREs with rhizobia can also improve nodulation (Preyanga et al., 2021). For instance, *Serratia* strain 33GS and R6 increased the relative abundance of *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium*, possibly indicating the indirect increase of nodulation by co-inoculation with NREs (Debnath et al., 2023). Accordingly, certain NREs may have the potential to positively influence nodule formation in *P. sativum*. Isolates *K. cowanii* LL3B1 and *P. chlororaphis* subsp. *aurantiaca* LL3B2 appeared to enhance nodule count, suggesting a potential synergistic relationship with *Rhizobium* spp. inoculants, similar to that described by Debnath et al. (2023). Although statistically significant results were not obtained

herein, the possibility for enhanced nodulation with NREs is intriguing. This rather unexplored group of bacteria could potentially be applied as co-inoculants to enhance N_2 fixation in legume crops, providing a valuable pathway to improve inoculant technology. Additionally, both of these organisms have previously been explored as PGP and biocontrol agents for other species (Morohoshi et al., 2017; Shi & Sun, 2017). Prior to this study the interactions of these species with *A. euteiches* were primarily unknown.

These characteristics suggests that the strategic assembly of beneficial microbial communities including both rhizobia and NREs could potentially strengthen plant defense against oomycete and various other plant pathogens. This may provide some room for optimizing current inoculant technology to include both an NRE biocontrol component and an N_2-fixing rhizobia component. However, additional knowledge on these specific interactions would be required to guarantee such an outcome.

5.3 Nutrients as a Factor in the Biocontrol of *Aphanomyces euteiches*

Interestingly, the strength of antagonism varied depending on the assay culture media, indicating the role of nutrient composition in shaping bacteria-oomycete interactions. Since competition for nutrients is a factor by which biocontrol occurs (Thomashow & Weller, 1996), this is not unexpected. It is also plausible that specific nutrients may shift the dynamic between these organisms, either amplifying or attenuating the production of secondary metabolites antagonistic towards *A. euteiches*. For instance, glucose and soytone supplementation increases phenazine production in *Pseudomonas* spp. (Yuan et al., 2008). This may explain observed differences in biocontrol activity in PDA, PDMA, PDYEMA, and YEMA seen in *P. chlororaphis* subsp. *aurantiaca* LL3B2. Specifically, different media that provide different nutrients may alter the interactions that are observed. Changing outcomes with varying culture media type may be

from nutrients shifting the bacteria-oomycete competitive dynamics. This observation implies that careful management of soil nutrient conditions, in tandem with inoculant technology, could potentially enhance protection from plant pathogens.

5.4 Additional Noteworthy Bacteria-Oomycete Interactions

In addition to observed antagonistic and non-antagonistic bacteria-oomycete interactions, two other interactions were of note. The first was a bacterial swarming of which the behavior varied depending on culture media. The second behavior defined here as a spreading behavior (Section 3.4.2, p. 25-27) occurred with all bacteria in the presence of *A. euteiches*, except for the antagonistic isolate *P. chlororaphis* subsp. *aurantiaca* LL3B2¸ and minimally in the case of *K. cowanii* LL3B1.

5.5 Swarming Mediated by Culture Media

Bacterial swarming was observed to vary depending on selected culture media. This was evidenced by *K. cowanii* LL3B1 migrating and enveloping an *A. euteiches* colony when grown on PDA and PDMA (Fig. 4.2, p. 40), however, not on YEMA (Fig. 4.3, p. 41). A potential explanation is that an abundance of nutrients is an important factor in bacterial swarming (Harshey, 2003). Comparatively, YEMA is relatively deprived of nutrients as opposed to PDA and PDMA.

However, this alone does not explain why *K. cowanii* LL3B1 would preferentially swarm towards and limit the growth of *A. euteiches*. In a constant environment, when individual bacteria swim using flagella, cells usually move in a random walk (Harshey, 2003); this is consistent with *K. cowanii* LL3B1 growing rather ubiquitously over culture media in absence of an *A. euteiches* colony. However, when swimming towards attractants or decreasing repellent concentrations, the random walk is biased in a preferred direction (Harshey, 2003). As opposed to swimming,

swarming is the movement of a group of bacteria (Williams and Schwarzhoff, 1978). It is possible that *A. euteiches* is an attractant for *K. cowanii* LL3B1 and induces selective swarming. However, *A. euteiches* may only be an attractant under certain nutrient conditions and/or specific nutrients regulate swarming. Ideally, nutrient conditions should be such that a greater extent of preferential swarming occurs towards *A. euteiches*¸ thereby reducing its ability to proliferate.

With respect to how nutrients effect bacterial swarming there are several considerations. First, the concentration of agar in culture media is critical for swarming, with 0.5% to 2.0% being optimal (Harshey, 2003). The recipes used here contained approximately 1.5% concentration of agar and were likely not a limiting factor. Moreover, swarming is often not observed in organisms on minimal media unless casamino acids are added (Eberl et al., 1996). Likely, this reflects the high metabolic cost of flagellar synthesis (Harshey, 2003).

It may be the case that *K. cowanii* is antagonistic towards *A. euteiches* through chitinase or cellulase activity. In other studies, this bacterium demonstrates cellulase and chitinase production (Lirio et al., 2020), which likely implies some capacity to break down chitin in the cell wall of *A. euteiches*. The preferential movement of *K. cowanii* LL3B1 towards *A. euteiches* along with production of extracellular enzymes known to break down the cellular components of *A. euteiches* may be indicative of predation.

There are several predatory strategies that may constitute a predatory relationship. These could either be epibiotic, wherein predators consume prey from the outside, or endobiotic wherein predators consume prey from the inside (Pérez et al., 2016). It is unclear if *K. cowanii* LL3B1 constitutes an epibiotic or endobiotic predation strategy as confirmation would require microscopy. It is possible that a myxobacteria-like strategy is used where swarming groups produce a range of predatory products which decompose prey cells (Pérez et al., 2016). This

strategy also requires close proximity to prey cells, which stimulates predation via the production of lytic enzymes and secondary metabolites (Berleman et al., 2014), along with density dependent swarming and gliding motility (Rosenberg et al., 1977). It is clear that for *K. cowanii* LL3B1 lytic enzymes are involved and close proximity to the surface of *A. euteiches* is necessary, which may indicate a myxobacteria-like predation strategy.

5.6 *Aphanomyces euteiches* Hyphae as Migratory Pathways for Nodule Endophytes

For soil-dwelling bacteria, air-filled pores in soil are a large barrier to movement (Wick et al., 2007). However, certain bacteria, such as *Pseudomonas putida* PpG7, can traverse pore spaces using hyphae of the oomycete *Pythium ultimum* (Wick et al., 2007). *Burkholderia terrae* BS001 uses a combination of flagella and type IV pili to co-migrate with fungal hyphae through soil (Yang et al., 2017). Additionally, *Burkholderia* can co-migrate with multiple fungal species and the ascomycetic hyphae of *Fusarium oxysporum* Fo47 (Warmink and van Elsas, 2009). This may explain the extensive spreading behavior observed in *Burkholderia* strain SU2B2 in the presence of *A. euteiches* (Fig. 4.3 p. 41).

Similarly, rhizobia are motile via flagella and type IV pili (Krehenbrink and Downie, 2008; Aroney et al., 2021). Flagella-based motility enables movement along root surfaces for more effective colonization (Simons et al., 1996; Gao et al., 2016). Consequently, it would not be surprising that rhizobia can also migrate along the surface of *A. euteiches* oomycetic hyphae. To establish symbiosis with a legume, a free-living rhizobia must successfully migrate from the soil environment to the host root to initiate the infection process. Movement along hyphal highways connected to legumes, such as that of *A. euteiches*, would be an evolutionarily advantageous method for rhizobia to ultimately reach their host plant. With respect to the observed spreading

behavior in Rhizobiaceae family bacteria (Fig. 4.3, p. 41), rhizobia may use the surface of *A. euteiches* hyphae as modes of transport.

5.7 Comments on Gnotobiotic Growth Pouch Experiments

In the third growth pouch experiment, the uninoculated control developed nearly no nodules and virtually zero relative N fixation (Figs. 4.12 and 4.13, p. 47-49), indicating relatively sterile conditions. However, the N+ uninoculated control developed many nodules and fixed a relatively high quantity of N. Given that one result indicates sterile conditions and the other indicates non-sterile conditions these observations seem contradictory. There is, however, an alternative explanation, which may involve an interaction between low levels of nitrate and endophytic rhizobial populations. Ai et al. (2023) surface-sterilized *Sophora davidii* seeds and analyzed the diversity of the seed endophytic community and found rhizobia at lower abundances compared with other microbiota. It was also found that these rhizobia can vertically transmit from the seed microbiome to nodules (Ai et al., 2023). In addition, rhizobia have been found as seed endophytes in *Cicer arietinum* L. (Laranjeira et al., 2022).

Nodulation in *P. sativum* can either be inhibited by ammonium supplementation or stimulated with appropriately low concentrations (Bollman and Vessey, 2006). Given the likely presence of rhizobia in *P. sativum* seed endophytic communities associated with the seed microbiome, and the capability of mineral N source to stimulate nodulation, it would not be out of the question that these two factors may have influenced the unexpected nodulation result from the third growth pouch experiment. In comparison, the second growth pouch experiment received a nitrate dose fifteen times higher than that of the third and resulted in much lower nodulation. Therefore, future growth pouch experiments should assess biocontrol inoculant performance in the presence of varying concentrations of N, as there is likely an interaction with respect to N

concentration. Here, inoculants were not combined with N, and it is unknown how results would have changed if this had been done. For this reason, the presented results of rhizobia and NRE as inoculants for *P. sativum* can only be fairly compared to the uninoculated N-free control group.

Despite attempting to control sources of contamination, root rot still developed during the growth pouch experiments. The root rot was confirmed to consist of *Alternaria* sp., *Fusarium redolens*, and *Fusarium solani*. As a result, direct conclusions on the *in vivo* effectiveness of isolates as inoculants on *A. euteiches* disease severity cannot yet be made. There is a possibility that these pathogens are able to proliferate underneath the seed coat and evade a standard seed surface-sterilization. A literature search on this topic was unable to provide any documents which evaluated this possibility, specifically in *P. sativum*.

As a consequence of seed endophytic communities, true gnotobiotic experiments involving *P. sativum* are difficult, if not impossible, to achieve.

5.8 Closing Remarks

Why do rhizobia appear to lack antagonistic properties towards *A. euteiches*? Within the context of the results of this study, a few possible conclusions arise. Possible reasons may include: (1) *Rhizobium* spp. antagonism towards *A. euteiches* is strain specific and uncommon; (2) lack of antagonism is a result of biochemical differences between *A. euteiches* and other phytopathogens within the PRRC; (3) *Rhizobium* spp. associated with *P. sativum* have different antagonistic properties than those isolated from other plants or soil; (4) the concentration of rhizobia in the dual plate assay was too low to elicit responses; and (5) from an ecological perspective, if it is the case that rhizobia use *A. euteiches* as modes of transport to legumes as part of their life cycle, it would be disadvantageous for rhizobia to develop antagonistic properties.

Moreover, a comprehensive understanding of the nutrient dependent behavior of antagonistic bacterial isolates would be invaluable. Future research should delve deeper into the specifics of how nutrient composition influences bacteria-oomycete interactions. Identifying key nutrients or conditions that alter the antagonistic effects of bacteria towards a plant pathogen could potentially lead to more effective strategies for agricultural systems. These findings could be applied to inoculant technology, leading to a more effective strategy for mitigation of crop damage from plant pathogens.

As closing remarks, the potential synergies observed between different bacterial isolates and *Rhizobium* spp. warrant further exploration. Future studies should aim to better define these relationships and their impact on nodule formation and N_2 fixation in legume crops. Similarly, the possibility of employing antagonistic bacteria as protective inoculants should be pursued in greater depth, with future research focusing on examining these effects in field trials to determine optimal application strategies. Furthermore, potential interactions between seed endophytic communities, including pathogens comprising the PRRC, and varying concentrations of mineral N may be of interest. In addition, future research should look to explore the multi-functionality of inoculant technology beyond N_2 fixation and aim to understand how best to leverage indigenous nodule-associated bacteria, including NREs for broader crop protection. As a whole, these approaches will be crucial for developing more effective mitigation of phytopathogens in a dynamically evolving agricultural landscape.

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APPENDIX A: TRAP PLANT ISOLATE INFORMATION

Table A.1 Isolates obtained from *P. sativum* trap plant nodules and their age at harvest.

Host Plant Age (days)	Isolate Code
42	LL3B1, LL3B2, MJ7A, LL1B, SU9A, SU9B, MJ12A, MJ12B, SPG5B, SU1B, SU1A, LL3A, MJ7B, SU6B, SU6A, SPG2B, MJ4A, MJ4B, SPG1A, SPG1B, SPG2A, SPG5A, SPG7A, LL4A, LL4B
47	LL1A, SU2B1, SU2B2, MJ5A, MJ5B, MJ8A, MJ8B, MJ10A, MJ10B, SPG6A, SPG6B, SPG8A, SPG8B, SPG4, SU4A1, SU5B, SU5A, SU4B, SU2A, SU7A, SU7B, LL7A, LL7B, LL6A, LL6B, LL2A, LL2B, SU4A2
63	SPG10B, SPG9B, SU3A, SU3B, MJ3A, MJ3B, MJ9A, MJ2B, MJ9B, MJ1B, LL10B, LL5B, LL5A, LL8A, LL8B, MJ6A, MJ6B

APPENDIX B: RECIPES

***Pisum sativum* trap plants**

Prepare two separate 4 L solutions in a volumetric flask, one containing all the micronutrients, and one containing all the macronutrients. These quantities are made such that 20 mL of each solution is added to 500 g of soil. Mix adequately with a magnetic stir bar.

Table B.1 Recipe for modified N-free Hoagland's Solution.

Element	Form	Mass (g) / 4 L H_2O
	Micronutrients	
Mo	$Na_2MoO_4 \cdot 2H_2O$	0.15134
B	H_3BO_3	0.858
Mn	$MnSO_4 \cdot H_2O$	1.538
Zn	$ZnSO_4 \cdot 7H_2O$	1.759
Cu	$CuSO_4 \cdot 5H_2O$	0.2358
	Macronutrients	
P	$Ca(H_2PO_4)_2$	11.334
K	K_2SO_4	17.289
S	KCl	24.36

***Pisum sativum* in growth pouches**

Table B.2 Recipe for 0.25x sterile N-Free nutrient solution (Broughton & Dilworth, 1971).

Stock Solution	Chemical	g/liter
1	$CaCl_2$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7 \circ 3H_2O$	6.7
	$MgSO_4 \circ 7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4 \circ H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4 \circ 7H_2O$	0.288
	$CuSO_4 \circ 5H_2O$	0.100
	$CoSO_4 \circ 7H_2O$	0.056
	$Na_2MoO_2 \circ H_2O$	0.048

After making stock solutions:

1. Add 5 mL of each stock solution to 0.5L deionized water.
2. Add 0.25g of KNO_3 to 0.5 L deionized water in separate jar for negative controls.
3. Adjust pH 6.6-6.8 before autoclaving with 0.1 M NaOH.
4. Dilute to 10 L after autoclaving.
5. Homogenize with magnetic stirrer before giving to plants.

Water with 20 mL of the final sterile nutrient solution every second day. Negative controls instead receive 20 mL of 0.05% (0.5g/L) KNO_3.

Recipes for PDA, PDMA, PDYEMA, YEMA, and CRYEMA

Tables B.3 to B.7 Recipes for culture media used in this study.

PDA Recipe

Ingredient	Grams per liter H_2O
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
potato dextrose	24
agar	15

PDMA Recipe

Ingredient	Grams per liter H_2O
mannitol	10
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
potato dextrose	24
agar	15

PDYEMA Recipe

Ingredient	Grams per liter H_2O
mannitol	10
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
yeast extract	0.4
potato dextrose	24
agar	15

YEMA Recipe

Ingredient	Grams per liter H_2O
mannitol	10
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
yeast extract	0.4
agar	15

CRYEMA Recipe

Ingredient	Grams per liter H_2O
mannitol	10
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
yeast extract	0.4
agar	15
Congo red solution	10 mL of 0.25g/100 mL solution

APPENDIX C: ISOLATE CULTURE MEDIA COMPATIBILITY DATA

Table C.1 Growth on different media after 5 d when incubated inverted in the dark at 23°C for isolates obtained from the nodules of field collected plants *L. venosus, L. culinarus,* and *P. sativum*.

Isolate Code	1/10 TSA	PDA	PDA Spread*	PDMA	PDYEMA	YEMA	CR Absorption
PP3	y			y		y	y
PP2C4	y	n	y	y		y	n
L1SN	y	n	y	y		y	n
FPD2	y	n	y	y		y	n
FP1	y	n	y	y		y	n
FP2	y	n	y	y		y	y
L1E3	y	n	y	y		y	n
LIE4	y	n	n	y		y	n
LIE2	y	n	y	y		y	n
PPC2	y	n	y	y		n	-
PPC1	y	n	y			n	-
LSA	y					y	n
PP2C3	y					y	n
FPD1	y					y	n
L2SB	y	n	y	y		y	n
PP1A1	y					n	-
PP1A2	y					y	n
FP2x	y					y	n
PP1A3	y	limited	no	y		y	y

*Demonstrated spreading behavior over *A. euteiches* after 5 d incubation period

Table C.2 Growth on different media after 5 d when incubated inverted in the dark at 23°C for isolates obtained from the nodules of *P. sativum* trap plants.

Isolate Code	Closest Identification	PDA	PDMA[†]	PDYEMA	YEMA	CR Absorption
LL3B2		y	+++++	y	y	n
LL3B1		y	+++++	y	y	mild – turns purple
MJ7A					y	n
LL1B					y	n
SU9A			++		y	n
SU9B					y	mild
MJ12A					y	n
MJ12B			+		y	n
SPG5B					y	n
SU1B					y	n
SU1A			+		y	n
LL3A			+++		y	n
MJ7B			+		y	n
SU6B					y	n
SU6A			+++		y	n
SPG2B					y	n
MJ4A			+		y	n
MJ4B					y	n
SPG1A					y	n
SPG1B			++		y	n
SPG2A					y	n
SPG5A					y	n
SPG7A			++		y	n
LL4A			++		y	n
LL4B			+++		y	n
LL1A			+		y	n
SU2B1			n		y	n
SU2B2		y	+++	y	y	n
MJ5A					y	n
MJ5B			+		y	n
MJ8A					y	n
MJ8B			+		y	n
MJ10A					y	n
MJ10B			+		y	n
SPG6A					y	n
SPG6B			+++		y	n
SPG8A					y	n

SPG8B	+++		y	n
SPG4	+		y	n
SU4A1			y	n
SU5B			y	n
SU5A	+++++		y	n
SU4B			y	n
SU2A	+++++	y	y	n
SU7A			y	n
SU7B			y	n
LL7A	++		y	n
LL7B			y	n
LL6A			y	n
LL6B	+++		y	n
LL2A	++		y	n
LL2B	+++		y	n
SU4A2	n		y	n
SPG10B	++		y	n
SPG9B	n		y	n
SU3A	++++		y	n
SU3B	++++		y	n
MJ3A			y	n
MJ3B			y	n
MJ9A	+++		y	mild
MJ2B			y	n
MJ9B	+		y	n
MJ1B	+		y	n
LL10B	+++		y	n
LL5B	+++		y	n
LL5A	++		y	n
LL8A	+++		y	n
LL8B	++++		y	n
MJ6A	++		y	n
MJ6B			y	n

† A plus sign (+) indicates media compatibility with (+++++) representing aggressive growth and (+) being the very limited growth while remaining visually evident. A (n) indicates that no growth was observed on the media over the incubation period, and (-) indicates that the isolate was not assessed for it's media compatibility.

Table C.3 Growth on different media after 5 d when incubated inverted in the dark at 23°C for isolates obtained from rhizobia culture collection.

Isolate Code	FAME ID	PDA	PDMA	PDMA Spread*	PDYEMA	YEMA
IC72	*Rhizobium* sp. *cicer*		n			
27A9	*Rhizobium leguminosarum* bv. *phaseoli*		y	y		
RP202-13	*Rhizobium* sp. *cicer*		y	y		
IC76	*Rhizobium* sp. *cicer*		n			
CP92	*Rhizobium* sp. *cicer*		n			
WPBS3644	*Rhizobium leguminosarum* bv. *phaseoli*		y	y		
CIAT632	*Rhizobium leguminosarum* bv. *phaseoli*		y	y		
9030	*Rhizobium leguminosarum* bv. *phaseoli*		y	y		
CIAT899	*Rhizobium leguminosarum* bv. *phaseoli*		y	y		
WPBS3622	*Rhizobium leguminosarum* bv. *phaseoli*					
9039	*Rhizobium leguminosarum* bv. *phaseoli*					
CIAT7230	*Rhizobium leguminosarum* bv. *phaseoli*					
CIAT7202	*Rhizobium leguminosarum* bv. *phaseoli*					
CIAT7100	*Rhizobium leguminosarum* bv. *phaseoli*		y			
CIAT2560	*Rhizobium leguminosarum* bv. *phaseoli*					
CIAT7136	*Rhizobium leguminosarum* bv. *phaseoli*					
LPNI-2	*Rhizobium leguminosarum* bv. *viciae*				y	y

Table C.4 Growth on different media after 5 d when incubated inverted in the dark at 23°C for antagonistic isolates obtained from Godebo et al. 2020.

Isolate Code	Closest Identification	PDA	PDMA	PDYEMA	YEMA
K-Be-H31		y	y		
K-H_f-H_2		y			

On all media types, growth rate of *A. euteiches* demonstrated a linear relationship with time (Fig. 7). The *A. euteiches* colony extends outwards at an average rate of 0.8 cm day^{-1} on YEMA. Conversely, growth on PDYEMA and PDMA was quicker with an average growth rate of 0.89 cm day^{-1} and 0.935 cm day^{-1}. Cores taken from the outer edge of 5 to 7 d *A. euteiches* pure cultures incubated at 23°C on YEMA, PDMA and PDYEMA transfer to new plates and grow normally. When sub-cultured on 5 to 8 d PDA, PDMA, PDYEMA, and YEMA *A. euteiches* reproduced successfully without noticeable differences.

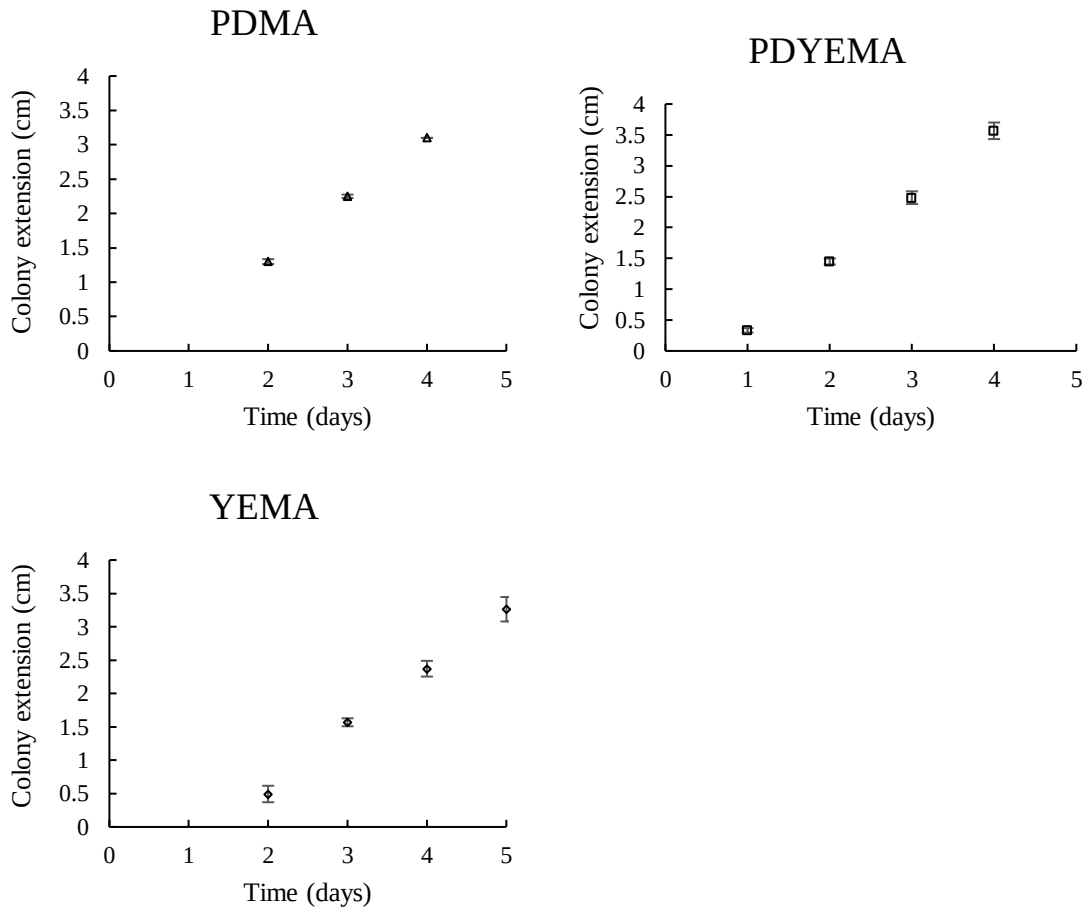

Figure C.5 *A. euteiches* colony extension over time on different culture media. Measurements are averages of 4 values recorded for each plate in all directions. Replicates for each: YEMA 4; PDMA 1; PDYEMA 3. Error bars are standard error.

APPENDIX D: DUAL PLATE BIOASSAY INFORMATION

Table D.1 All isolates and replication for each culture media. A total of four positive controls were evaluated (Godebo et al. 2020), along with 17 culture collection isolates, 42 soil trap isolates, and 13 isolates from field collected specimens.

Isolate Code*	Source	Media Replicates†			
		PDA	PDMA	PDYEMA	YEMA
K-Be-H_3	soil (Godebo et al. 2020)	2+1+3	1+3	-	-
K-H_f-H_2	soil (Godebo et al. 2020)	6	6	-	-
PCB2-6	soil (Godebo et al. 2020)	3	3	-	-
K-CB_2	soil (Godebo et al. 2020)	3	3	-	-
IC72	nodule bacteria, culture collection	1	1	-	-
27A9	nodule bacteria, culture collection	1	2	-	-
RP202-13	nodule bacteria, culture collection	1	2	-	-
IC76	nodule bacteria, culture collection	1	2	-	-
CP92	nodule bacteria, culture collection	1	2	-	-
WPBS3644	nodule bacteria, culture collection	1	1	-	1
CIAT632	nodule bacteria, culture collection	1	1+1	-	-
9030	nodule bacteria, culture collection	1	1	-	-
CIAT899	nodule bacteria, culture collection	1	1	-	1
WPBS3622	nodule bacteria, culture collection	-	-	-	1
9039	nodule bacteria, culture collection	-	-	-	1
CIAT7230	nodule bacteria, culture collection	-	-	-	1
CIAT7202	nodule bacteria, culture collection	-	-	-	1
CIAT7100	nodule bacteria, culture collection	1	1	-	1
CIAT2560	nodule bacteria, culture collection	-	1	-	-
CIAT7136	nodule bacteria, culture collection	-	1	-	-
LPNI-2	nodule bacteria, culture collection	-	-	6	6
LL3B2	*P. sativum* trap plant root nodule	1+3+3	1+3	3+3	1+3
LL3B1	*P. sativum* trap plant root nodule	1+3+3	1+3	3+3	1+3
MJ7A	*P. sativum* trap plant root nodule	1	-	-	-
LL1B	*P. sativum* trap plant root nodule	1	-	-	-
SU9A	*P. sativum* trap plant root nodule	-	-	-	-
SU9B	*P. sativum* trap plant root nodule	1	-	-	-
MJ12A	*P. sativum* trap plant root nodule	1	-	-	-
MJ12B	*P. sativum* trap plant root nodule	-	-	-	-
SPG5B	*P. sativum* trap plant root nodule	1	-	-	-
SU1B	*P. sativum* trap plant root nodule	1	-	-	-
SU1A	*P. sativum* trap plant root nodule	-	-	-	-
LL3A	*P. sativum* trap plant root nodule	-	-	-	1

MJ7B	*P. sativum* trap plant root nodule	-	-	-	-
SU6B	*P. sativum* trap plant root nodule	1	-	-	-
SU6A	*P. sativum* trap plant root nodule	-	1	-	-
SPG2B	*P. sativum* trap plant root nodule	-	-	-	-
MJ4A	*P. sativum* trap plant root nodule	-	-	-	-
MJ4B	*P. sativum* trap plant root nodule	1	-	-	-
SPG1A	*P. sativum* trap plant root nodule	1	-	-	-
SPG1B	*P. sativum* trap plant root nodule	-	-	-	1
SPG2A	*P. sativum* trap plant root nodule	1	-	-	-
SPG5A	*P. sativum* trap plant root nodule	-	-	-	-
SPG7A	*P. sativum* trap plant root nodule	-	-	-	-
LL4A	*P. sativum* trap plant root nodule	-	-	-	-
LL4B	*P. sativum* trap plant root nodule	-	-	-	-
LL1A	*P. sativum* trap plant root nodule	-	-	-	-
SU2B1	*P. sativum* trap plant root nodule	-	-	3	-
SU2B2	*P. sativum* trap plant root nodule	1+3	3	3+3	1+3
MJ5A	*P. sativum* trap plant root nodule	1	-	-	-
MJ5B	*P. sativum* trap plant root nodule	-	-	-	-
MJ8A	*P. sativum* trap plant root nodule	1	-	-	-
MJ8B	*P. sativum* trap plant root nodule	-	-	-	-
MJ10A	*P. sativum* trap plant root nodule	-	1	-	-
MJ10B	*P. sativum* trap plant root nodule	-	-	-	-
SPG6A	*P. sativum* trap plant root nodule	1	-	-	-
SPG6B	*P. sativum* trap plant root nodule	-	-	-	1
SPG8A	*P. sativum* trap plant root nodule	-	1	-	-
SPG8B	*P. sativum* trap plant root nodule	-	-	-	1
SPG4	*P. sativum* trap plant root nodule	-	-	3	-
SU4A1	*P. sativum* trap plant root nodule	1	-	-	-
SU5B	*P. sativum* trap plant root nodule	-	1	-	-
SU5A	*P. sativum* trap plant root nodule	-	-	-	1
SU4B	*P. sativum* trap plant root nodule	-	1	-	-
SU2A	*P. sativum* trap plant root nodule	-	-	3	1+3
SU7A	*P. sativum* trap plant root nodule	-	1	-	-
SU7B	*P. sativum* trap plant root nodule	-	-	-	-
LL7A	*P. sativum* trap plant root nodule	-	-	-	-
LL7B	*P. sativum* trap plant root nodule	-	-	-	-
LL6A	*P. sativum* trap plant root nodule	-	1	-	-
LL6B	*P. sativum* trap plant root nodule	-	-	-	1
LL2A	*P. sativum* trap plant root nodule	-	-	-	-
LL2B	*P. sativum* trap plant root nodule	-	-	-	1

SU4A2	*P. sativum* trap plant root nodule	-	-	3	-
SPG10B	*P. sativum* trap plant root nodule	-	-	-	1
SPG9B	*P. sativum* trap plant root nodule	-	-	-	-
SU3A	*P. sativum* trap plant root nodule	-	-	-	1
SU3B	*P. sativum* trap plant root nodule	-	-	-	-
MJ3A	*P. sativum* trap plant root nodule	-	-	-	-
MJ3B	*P. sativum* trap plant root nodule	-	-	-	-
MJ9A	*P. sativum* trap plant root nodule	-	-	-	-
MJ2B	*P. sativum* trap plant root nodule	-	-	-	-
MJ9B	*P. sativum* trap plant root nodule	-	-	-	-
MJ1B	*P. sativum* trap plant root nodule	-	-	-	-
LL10B	*P. sativum* trap plant root nodule	-	-	-	1
LL5B	*P. sativum* trap plant root nodule	-	-	-	-
LL5A	*P. sativum* trap plant root nodule	-	1	-	-
LL8A	*P. sativum* trap plant root nodule	-	1	-	-
LL8B	*P. sativum* trap plant root nodule	-	-	-	1
MJ6A	*P. sativum* trap plant root nodule	-	-	-	1
MJ6B	*P. sativum* trap plant root nodule	-	-	-	-
PP3	*L. venosus* nodule	1	-	-	1
PP2C4	*L. venosus* nodule	1	-	-	1
L1SN	*Lens culinarus* nodule	1	-	-	1
FPD2	*P. sativum* nodule	1	-	-	1
FP1	*P. sativum* nodule	1	-	-	1
FP2	*P. sativum* nodule	1	-	-	1
L1E3	*L. culinarus* nodule	1	-	-	1
LIE4	*L. culinarus* nodule	1	1	-	-
LIE2	*L. culinarus* nodule	1	-	-	-
PPC2	*L. venosus* nodule	1	-	-	-
PPC1	*L. venosus* nodule	1	-	-	-
LSA	*L. culinarus* nodule	-	-	-	-
PP2C3	*L. venosus* nodule	-	-	-	-
FPD1	*P. sativum* nodule	-	-	-	-
L2SB	*L. culinarus* nodule	1	-	-	-
PP1A1	*L. venosus* nodule	-	-	-	-
PP1A2	*L. venosus* nodule	-	-	-	-
FP2x	*P. sativum* nodule	-	-	-	-
PP1A3	*L. venosus* nodule	1	-	-	-

* Isolate codes were used as an identifier prior to proper identification

† Replicate plates for each isolate and culture media type; replicate numbers are separated by a (+) sign to indicate repeated bioassays (e.g., 2+1 means 2 replicate plates in the first bioassay, then 1 replicate plate in a successive bioassay).

Table D.2 Isolate antagonism outcomes on different media towards *A. euteiches.*

Isolate Code*	Source	Closest Identification†	Antagonism§			
			PDA	PDMA	PDYEMA	YEMA
K-Be-H_3	soil (Godebo et al. 2020)	*Lysobacter gummosus*	+++++	+		
K-H_f-H_2	soil (Godebo et al. 2020)	*Lysobacter capsici*	+++++			
LL3B2	*P. sativum* trap plant root nodule	*Pseudomonas chlororaphis* subsp. *aurantiaca*	++	+	-	+
SU2B2	*P. sativum* trap plant root nodule	*Burkholderia* sp.	+++		+	-
PCB2-6	soil (Godebo et al. 2020)	*Bacillus cereus*	++			
K-CB_2	soil (Godebo et al. 2020)	*Bacillus cereus*	++			
LL3B1	*P. sativum* trap plant root nodule	*Kosakonia cowanii*	-¶	-¶	-¶	-

* Isolate codes were used as an identifier prior to proper identification

† Closest identification was determined from the closest match with >97% similarity when sequences were queried in the NCBI BLAST database

§ A plus sign (+) indicates the relative size of the zone of antagonism with (+++++) being the largest and (+) being the smallest while remaining visually evident. A (-) indicates that no zones of antagonism were observed on the media used within the assay. A blank cell means that the isolate was not evaluated on the respective culture media.

¶ Although no zone of antagonism was observed, swarming behavior resulted in strong antagonistic behavior towards *A. euteiches*, however, this did not occur on YEMA.

APPENDIX E: GROWTH POUCH EXPERIMENT DATA

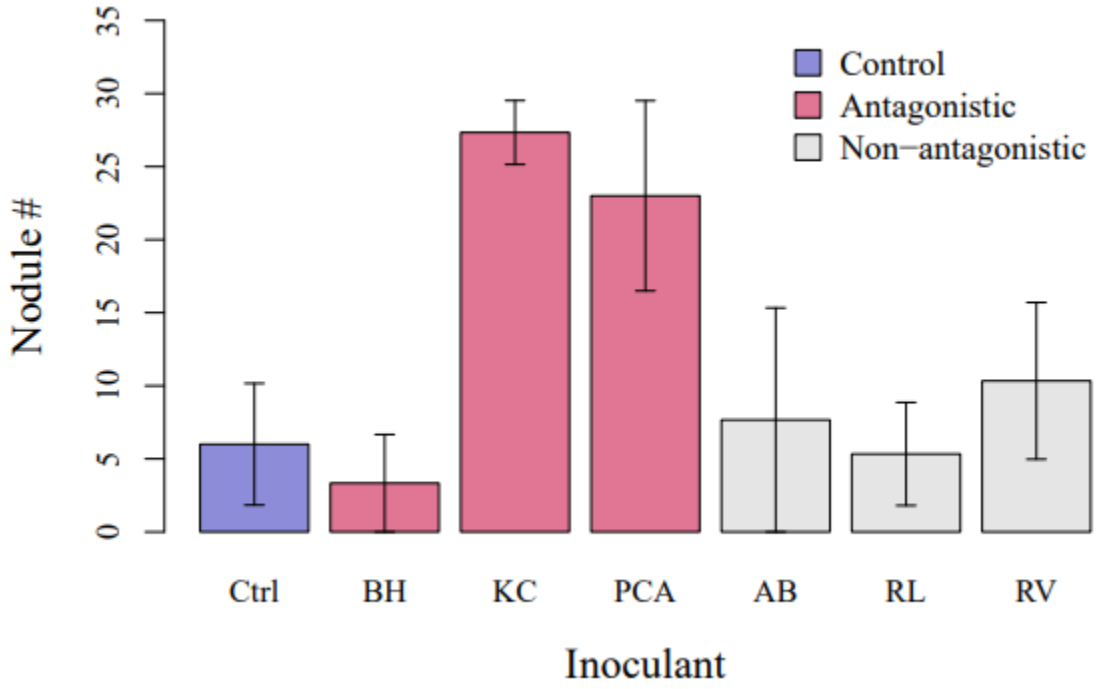

Figure E.1 *Pisum sativum* nodule count outcome for each inoculant: *Burkholderia* strain SU2B2 (BH), *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* strain LL8B (RL), and *Rhizobium leguminosarum* bv. *viciae* (RV). Control was uninoculated N-free (Ctrl). Error bars are standard error. Bars represent the mean of three replicates per inoculant with three *P. sativum* seeds per growth pouch. Data was from the second growth pouch experiment. No significant detected ($p < 0.05$).

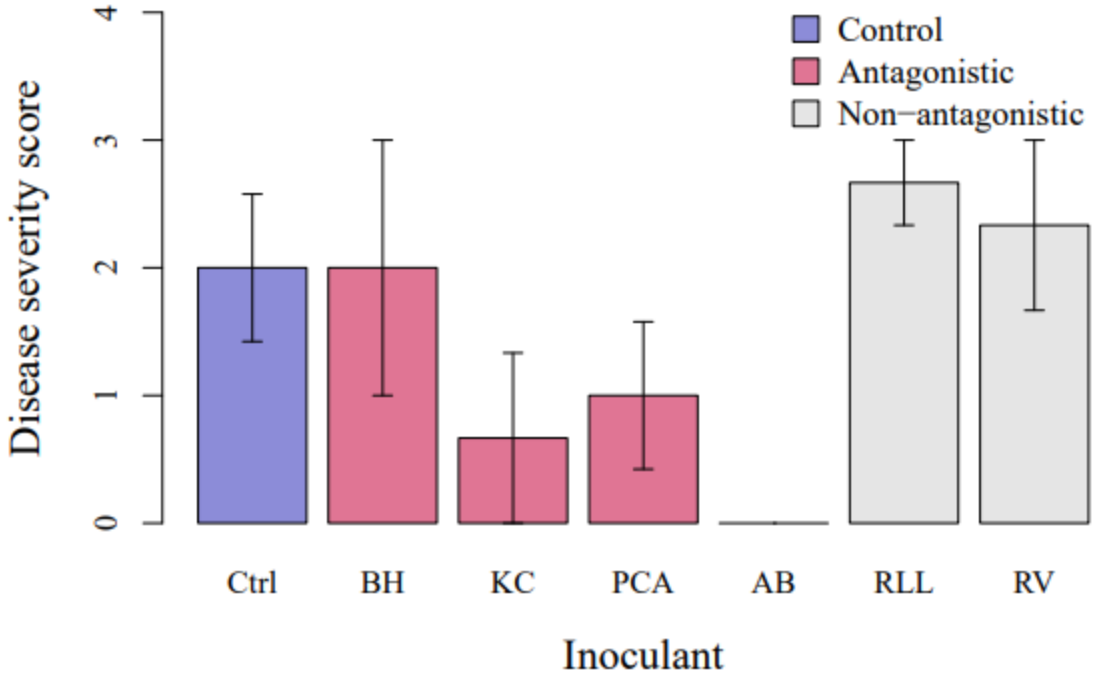

Figure E.2 *Pisum sativum* root rot disease severity outcomes after 5 wks for each inoculant: *Burkholderia* strain SU2B2 (BH), *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* strain LL8B (RLL), and *Rhizobium leguminosarum* bv. *viciae* (RV). Bars represent the mean of three replicates per inoculant. Error bars are standard error. Disease severity was scored following the procedure described by Wakelin et al. (2002). 0 = no symptoms; roots healthy and white. 1 = initial symptoms of root rot; discoloration, usually a light tan color, in sections. 2 = discoloration of most or all of the root system, usually still a tan color. 3 = advanced disease symptoms; extensive darkening and discoloration of the root system and extensive lesion formation. 4 = root entirely rotted/plant dead. Growth pouch positions were randomized. Data is from the second growth pouch experiment.

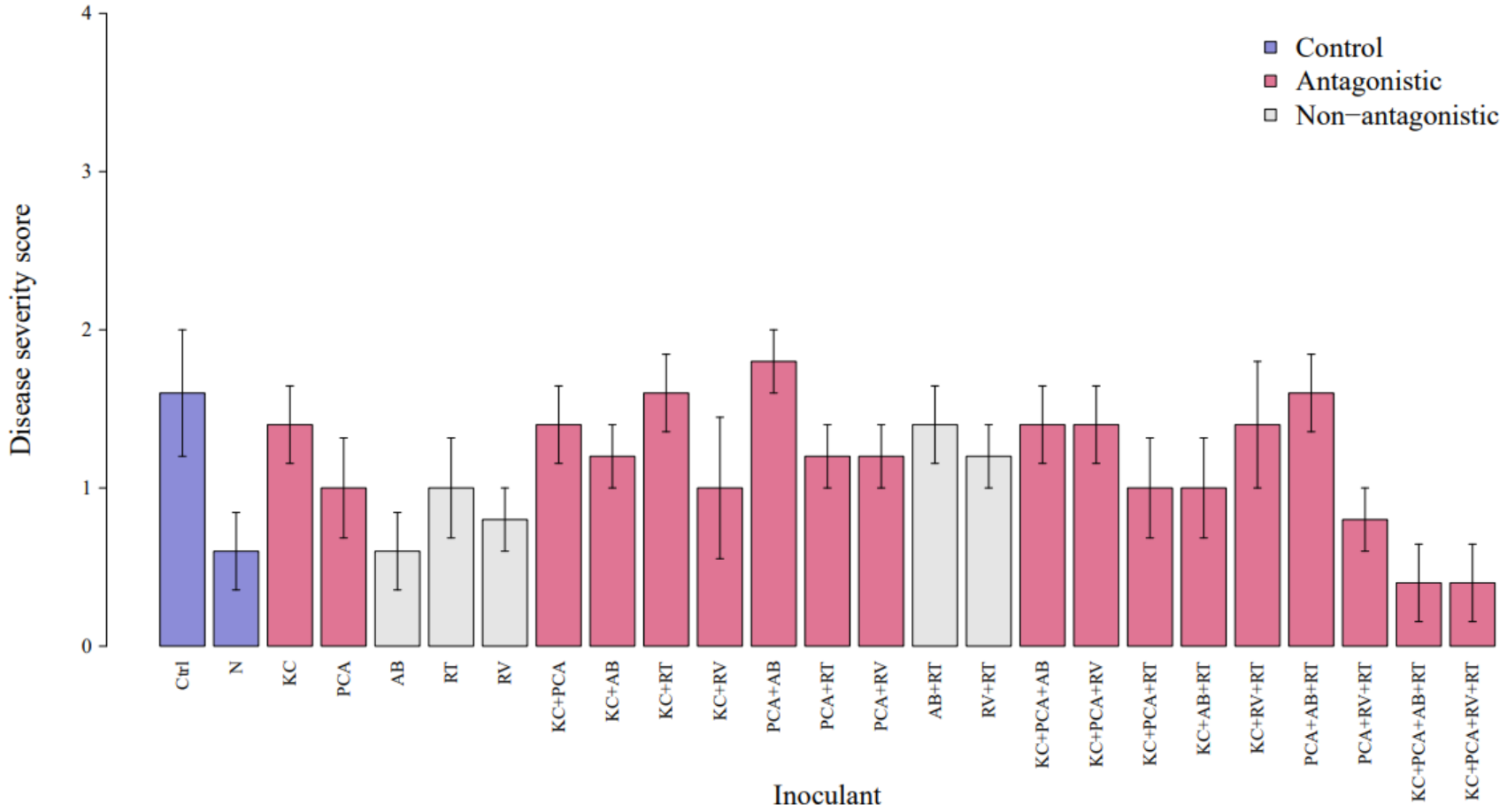

Figure E.3 *Pisum sativum* root rot disease severity outcomes after 30 d for each inoculant combination: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Controls were uninoculated N-free (Ctrl) and uninoculated supplemented with 0.05% KNO_3 (N). Error bars are standard error. Disease severity was scored blind following the procedure described by Wakelin et al. (2002). 0 = no symptoms; roots healthy and white. 1 = initial symptoms of root rot; discoloration, usually a light tan color, in sections. 2 = discoloration of most or all of the root system, usually still a tan color. 3 = advanced disease symptoms; extensive darkening and discoloration of the root system and extensive lesion formation. 4 = root entirely rotted/plant dead. Growth pouch positions were randomized. Data is from the third growth pouch experiment. No significant differences were detected ($p < 0.05$).

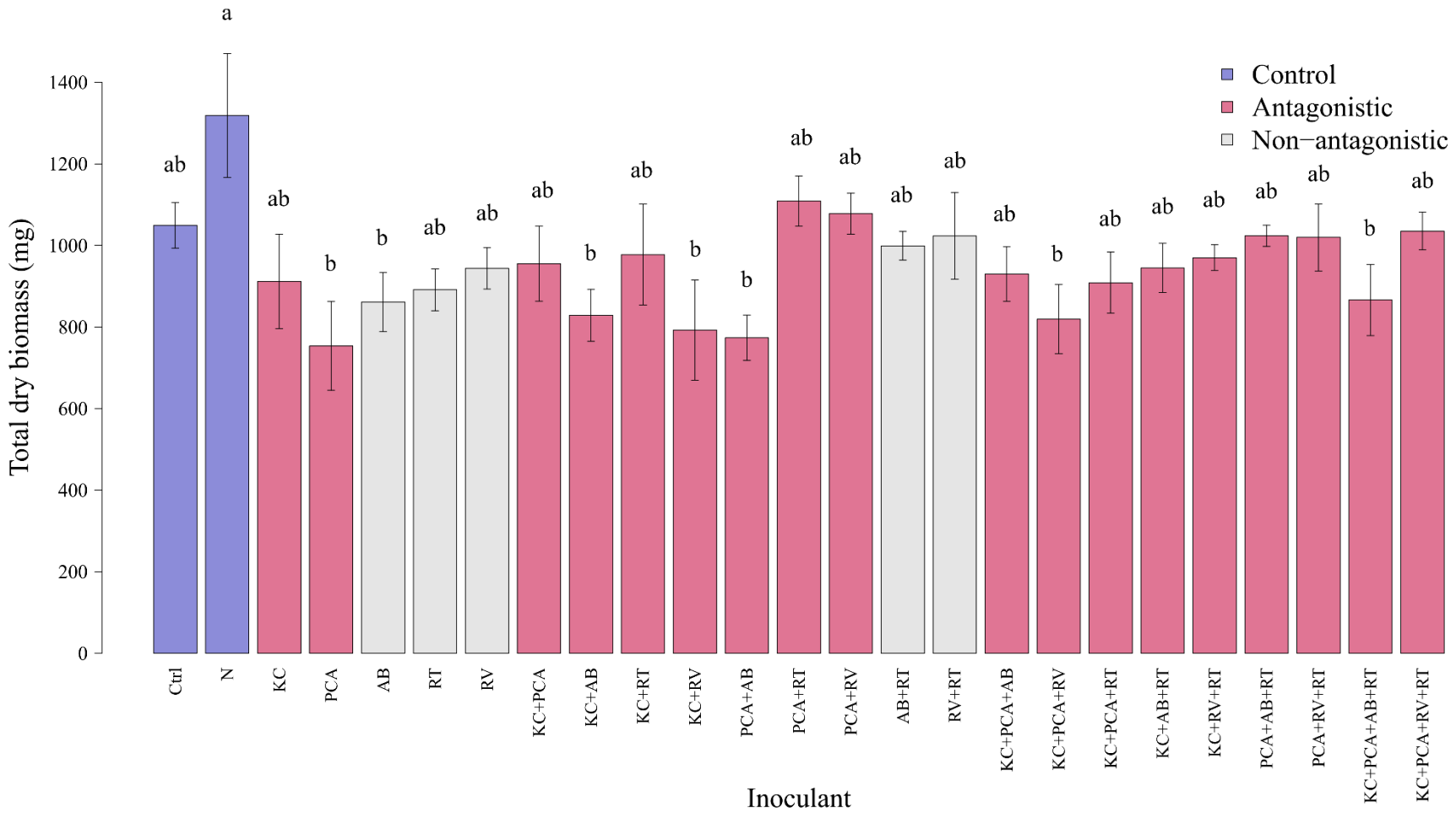

Figure E.4 *Pisum sativum* total dry biomass after 30 d for each inoculant combination: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Combinations are represented by isolate abbreviations separated by a plus (+) sign. Controls were uninoculated N-free (Ctrl) and uninoculated supplemented with 0.05% KNO_3 (N). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Data is from the third growth pouch experiment. Letters represent significant differences ($p < 0.05$).

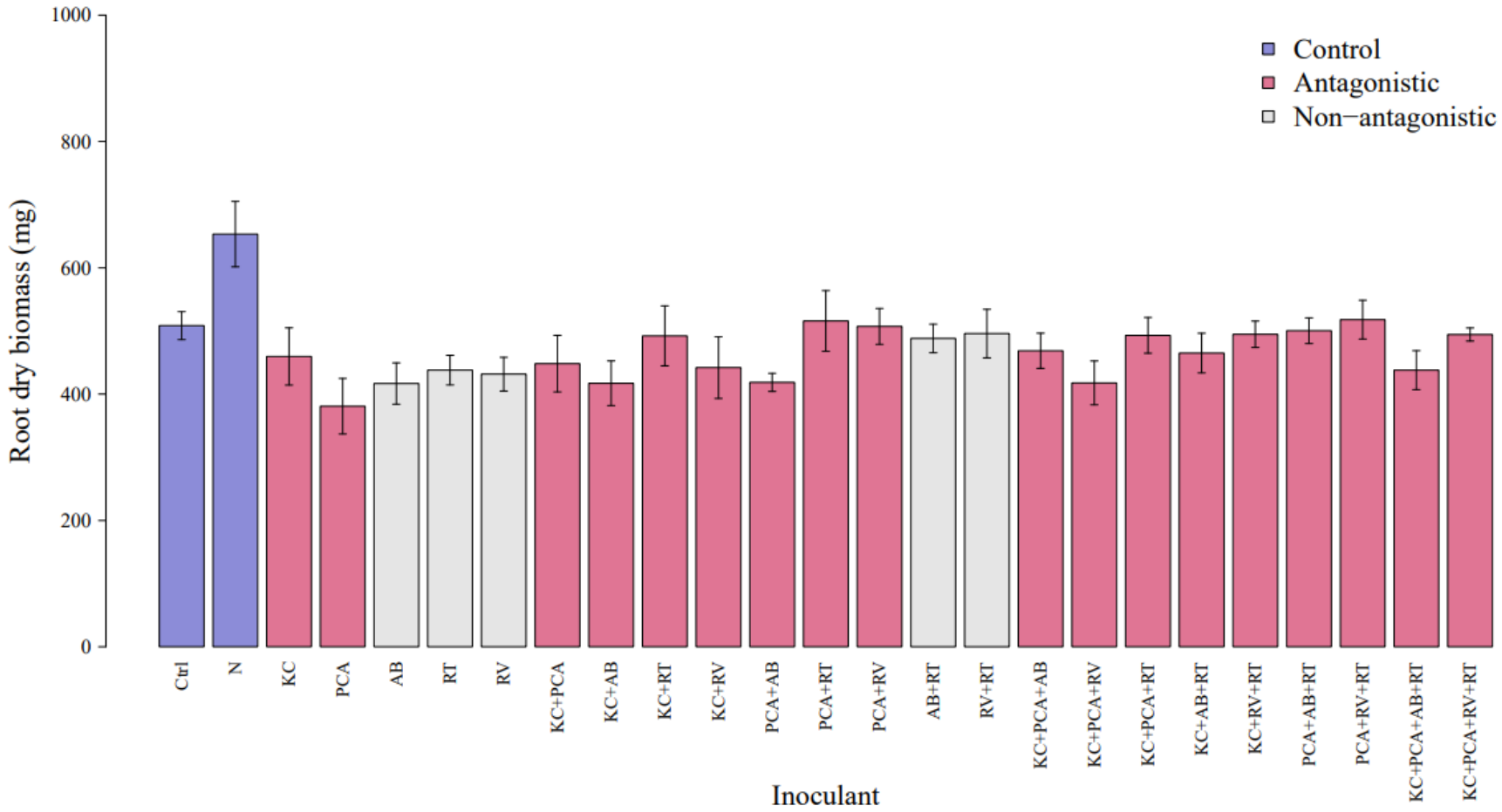

Figure E.5 *Pisum sativum* root dry biomass after 30 d for each inoculant combination: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Combinations are represented by isolate abbreviations separated by a plus (+) sign. Controls were uninoculated N-free (Ctrl) and uninoculated supplemented with 0.05% KNO_3 (N). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Data is from the third growth pouch experiment.

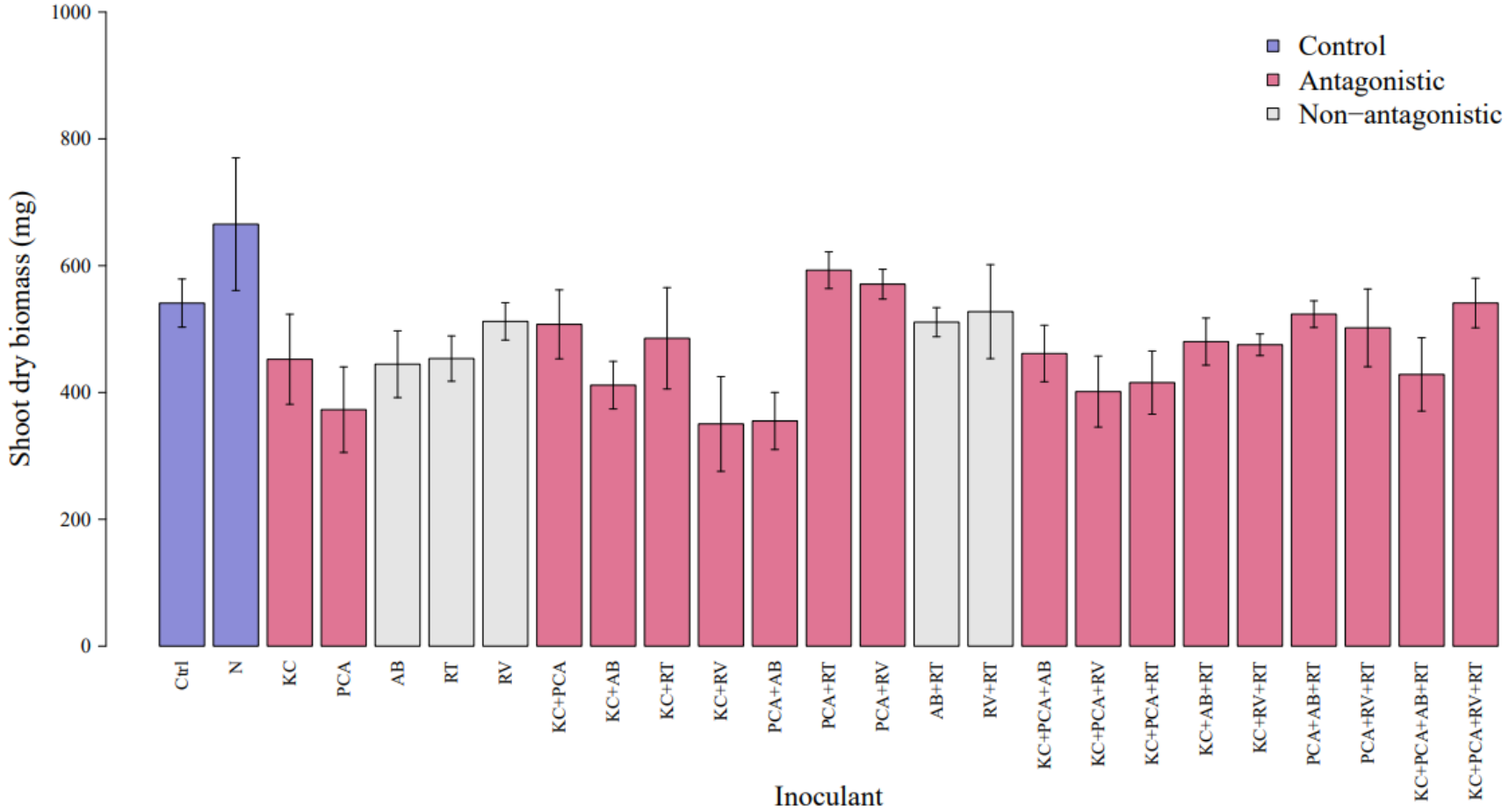

Figure E.6 *Pisum sativum* shoot dry biomass after 30 d for each inoculant combination: *Kosakonia co*wanii LL3B1 (KC), *Pseudomonas chlororaphis* subsp. *aurantiaca* LL3B2 (PCA), *Agrobacterium* strain SU2A (AB), *Rhizobium leguminosarum* bv. *trifolii* LPNI-2, and *Rhizobium leguminosarum* bv. *viciae* SPG10B (RV). Combinations are represented by isolate abbreviations separated by a plus (+) sign. Controls were uninoculated N-free (Ctrl) and uninoculated supplemented with 0.05% KNO_3 (N). Bars represent the mean of five replicates per inoculant treatment. One replicate is defined as a growth pouch with three *P. sativum* seeds. Error bars are standard error. Data is from the third growth pouch experiment.