Pseudomonas chlororaphis strain PA23 plant growth promotion characteristics and product formulation development.

By

Amanda Holden

A Thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Microbiology
University of Manitoba
Winnipeg

ABSTRACT

Pseudomonas chlororaphis PA23 is a biocontrol agent that exhibits plant growth promotion (PGP) potential. The focus of this research was to investigate the PGP capacity of PA23 either through drenching soil-grown plants with bacterial suspensions or by exposing plants to bacterial volatiles in enclosed split-plate assays. In soil experiments, canola and lettuce treated with PA23 and the *gacS* mutant showed no increase in biomass. However, plants exposed to bacterial volatiles were significantly larger. Liquid- and dry-based formulations were investigated for their impact on PA23 cell viability over the course of 1 year at 4^{o}C. Liquid formulations contained PA23 cells suspended in LB with one or more additives to protect against desiccation. For dry formulations, PA23 was cultured in nutritional, minimal or high salt media and lyophilized with lactose. While dry formulations maintained a relatively high viability over time, they were unable to protect canola from infection with *Leptosphaeria maculans.* Liquid formulations demonstrated better biocontrol properties but showed a dramatic loss of viability upon storage at 4^{o}C.

ACKNOWLEDGEMENTS

Firstly, I would like to express my utmost gratitude to my supervisor, Dr. Teresa de Kievit, for giving me the opportunity to work in her lab. Her dedication to helping students is truly inspiring. I am very thankful for the support and guidance she has provided me throughout my thesis, especially during challenging times. She has made me a better writer and pushed me to become a better researcher.

To Dr. Belmonte and his lab members, thank you for the kindness and generosity you all have given me throughout my thesis project. I am grateful for the knowledge and assistance you have given me during my plant experiments. Thank you for allowing me to use your lab and share your growth space. It was truly wonderful interacting with you all and your willingness to help was amazing.

I would like to thank Dr. Dilantha Fernando and his lab, for their generosity and providing me with canola seeds. Thank you for sharing your growth space and planting supplies, I am very grateful.

Finally, thank you to all of my lab mates, past and present. A special thank you to Munmun, for helping me with my plant assays, phenotypic assays and formulation preparation. Also, thank you to Nidhi, Akrm, Kelly, Grace, Elham, April and past summer students in the de Kievit lab, it has been an absolute pleasure to work and spend time with you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
AHL	acyl-homoserine lactone
ANR	anaerobic regulator
DAPG	2,4-diacetylphloroglucinol
Gac	global activator of antibiotic and cyanide
GB	glycine betaine
GC-MS	gas chromatography-mass spectrophotometry
HCN	hydrogen cyanide
IAA	indole acetic acid
ISR	induced systemic resistance
LB	lysogeny broth
M9	minimal media
NaCl	sodium chloride
PGP	plant growth promotion
PGPR	plant growth promoting rhizobacteria
PHZ	phenazine
PRN	pyrrolnitrin
PsrA	*Pseudomonas* sigma regulator A
QS	quorum sensing
RpoS	sigma factor
Rsm	regulator of secondary metabolites
SAR	systemic acquired resistance
TB	terrific broth
Wt	wild type

1. INTRODUCTION

1.1 The importance of canola to Canadian agriculture

Agriculture in Canada has developed into a key industry that helps sustain the economy through the global market. In fact, Canada is the 5th largest exporter of agricultural goods worldwide generating over $110 billion in gross domestic product annually (Agriculture and Agri-Food Canada, 2017). One of the most valuable crops grown in Canada is canola. In 2015, canola production yielded the largest cash return for farmers compared to any other major Canadian crop (Statistics Canada, 2016). Canada is the largest global exporter of canola, exporting over 90% of its canola; as such, the cash return is driven by increasing demand in global markets (USDA, 2015). This demand is forecasted to continue to increase over the next 10 years with the top consumers reported to be the United States and China (Statistics Canada, 2016). It is no surprise that land acreage dedicated to canola production will increase in the coming years; therefore, sustainability of this crop is crucial for the Canadian economy.

Canola is exported primarily as seed, meal and oil. At the time of harvest, seed is collected and crushed to yield two products: oil and meal. The oil contains a high degree of unsaturated fats with essential fatty acids for human health, such as alpha-linolenic acid and linoleic acid (CCGA, 2016). The protein-rich meal is used as feed for farm animals (CCGA, 2016). In addition, canola represents an efficient renewable source as biodiesel because it emits less greenhouse gases compared to fossil fuels (CCGA, 2016). Given the growing demand and wide range of uses for canola, it is important to maintain sustainable farming practices to support increased growth of this important Canadian crop.

1.2 *Brassica napus*

Canola is a variety of rapeseed that belongs to the *Brassica napus* genus. The variety that is grown in Canada today is quite different from the rapeseed grown during the early 1900's. Prior to the 1970's, it was generally accepted that the oil from rapeseed was inedible due to high levels of erucic acid and glucosinolates. Erucic acid, which remains in the oil when extracted from seed, has been shown to cause myocardial distress in rats (Guil, Rodríguez-García, and Torija 1997); accordingly, human consumption of erucic acid is not recommended. Moreover, the meal contains glucosinolates, which emit a sharp unsavoury flavour that is unpalatable (Casséus 2009). In the 1970's, scientists at the University of Manitoba used parental breeding methods to create a variety that resulted in negligible amounts of erucic acid (<2%) in the oil and glucosinolates (<30umoles) in the meal complying with nationally acceptable levels (Canadian Canola Council, 2017). The name canola was created from the prefix *Can-* to represent Canada and *ola-* a Latin word meaning oil (Canadian Canola Council, 2017).

B. napus is an annual or biannual plant that produces bright yellow flowers which are the reproductive centers for pod production and seed development. As shown in Figure 1.1, plant growth can be divided into two stages: the vegetative stage and the reproductive stage (Canola Council of Canada, 2017). The first part of the vegetative stage is germination of a newly sown seed. Once sufficient water has been absorbed, activated enzymes release food stores to develop a radicle root that sprouts from the seed (Canola Council of Canada, 2017). Emergence of the hypocotyl occurs about 4 to 15 days after seeding (Canola Council of Canada, 2017). Upon exposure to light, two heart-shaped cotyledons unfold and the plant is now called a seedling. Thereafter, the first true leaves develop that are wrinkled in appearance compared to the cotyledons which are smooth (Canola Council of Canada, 2017). As the stem elongates, buds

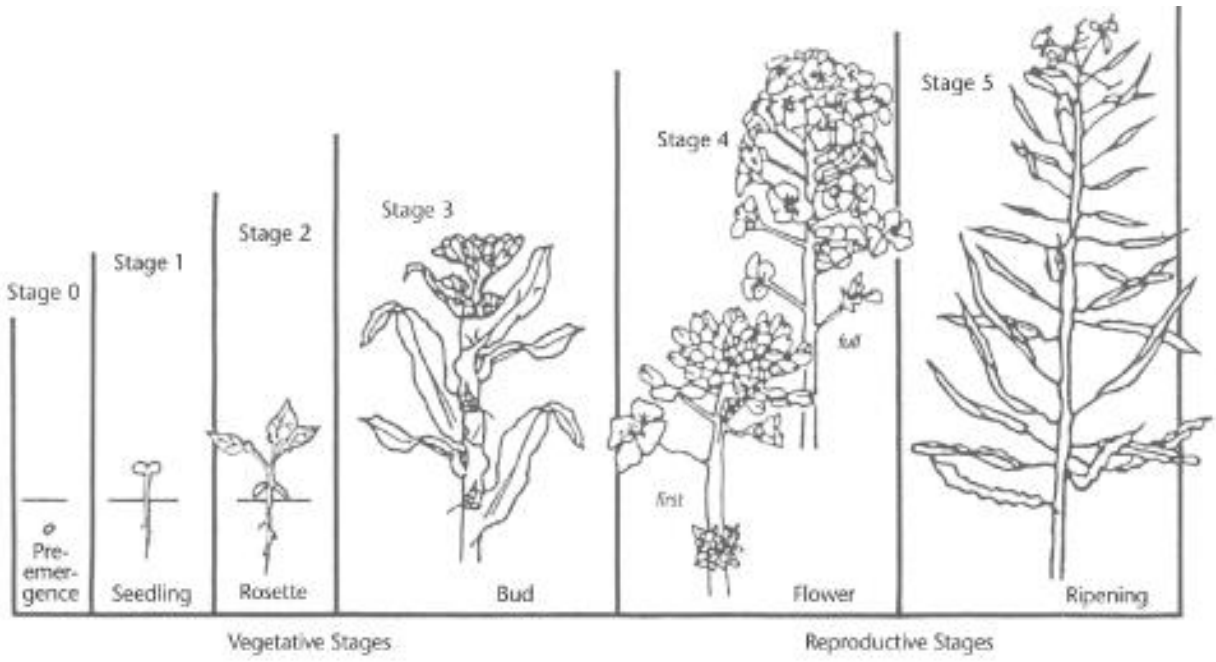


Figure 1.1 Developmental stages of canola plant growth. Reproduced from the Canola Council website. https://www.canolacouncil.org/canola-encyclopedia/crop-development/growth-stages/.

will form which will give rise to flowers, the reproductive structures. As flowers begin to bloom, fertilization through self-pollination occurs to initiate pod development (Canola Council of Canada, 2017). Once flowering has reached 30%, petals will start to wilt and fall off (Canola Council of Canada, 2017). It is during the flowering stage that canola is vulnerable to attack by fungal pathogens such as *Sclerotinia sclerotiorum* (Lib de Bary). Active flowering will continue until approximately 50% of flowers are open, after which the pods will elongate and the seeds will mature within the pod. From sowing to harvest it takes around 13 weeks for Westar canola plants to reach full maturity; however, this time frame is subject to change depending on the location, current environmental conditions and time of sowing. In Manitoba, canola from the *B. napus* variety can take anywhere from 10-17 weeks to reach maturity (Canola Council of Canada, 2017).

1.3 Disease management

Currently, there are three main practises used to prevent or reduce plant diseases caused by microbial pathogens: crop rotation, breeding for resistant cultivars and application of pesticides. Selecting which approach to employ depends on the epidemiology of the pathogen. Crop rotation can revive the nutritional content of the soil by cycling crops with nitrogen fixers, such as legumes, but more importantly it can help to reduce persisting diseases (Ghorbani et al. 2008). For example, rotating a crop with a non-host plant may prevent a reoccurring infection, especially if the pathogen has a short saprophytic life cycle. However, for pathogens that survive longer in the soil, breeding resistant cultivars may be more effective for managing disease. One challenge in generating a resistant cultivar is the complexity of genes involved in the infection

process (Gururani et al. 2012). A second limitation is that pathogens have the potential to adapt, which may enable them to infect disease-resistant cultivars. An alternative approach to disease management is the application of pesticides. There are, however, major concerns regarding the negative impact of these chemicals on the environment and on human health. Thus, there is an urgent need for safer alternatives that can suppress even the most resilient pathogens.

1.4 *Sclerotinia sclerotiorum*

Necrotrophic in nature, *S. sclerotiorum* can infect over 400 host plants by a complex network of infection pathways. In canola, *S. sclerotiorum* causes stem rot that can lead to a complete loss of crop viability. This fungus is a saprophytic pathogen with the ability to survive up to 5 years or more in soil through production of resistant melanised bodies called sclerotia (figure 1.2) (Link and Johnson, 2012). When spring brings continuous moisture into the soil apothecia emerge, which are the reproductive structures that produce ascospores (Hegedus and Rimmer 2005). The spores are released into the air and land on senescing petals during the 30-50% flowering stage of canola development. The ascospores use the decaying matter to generate an appressorium, the structure required for penetrating the cuticle. Once penetration has occurred, a vesicle is formed that is released into the cuticle. This vesicle exudes growing hyphae that mediate infection and degrade cell tissues. In addition to degradative enzymes which help facilitate infection, oxalic acid plays a critical role in the infection process (Hegedus and Rimmer 2005). Oxalic acid secretion creates an acidic environment in host tissues that enhances activity of degradative enzymes. More importantly, oxalic acid sequesters calcium from plant cell walls making the cells more vulnerable to degradation by fungal enzymes (Dutton and Evans, 1996). As cell degradation progresses, plant tissue dies and necrotic lesions become

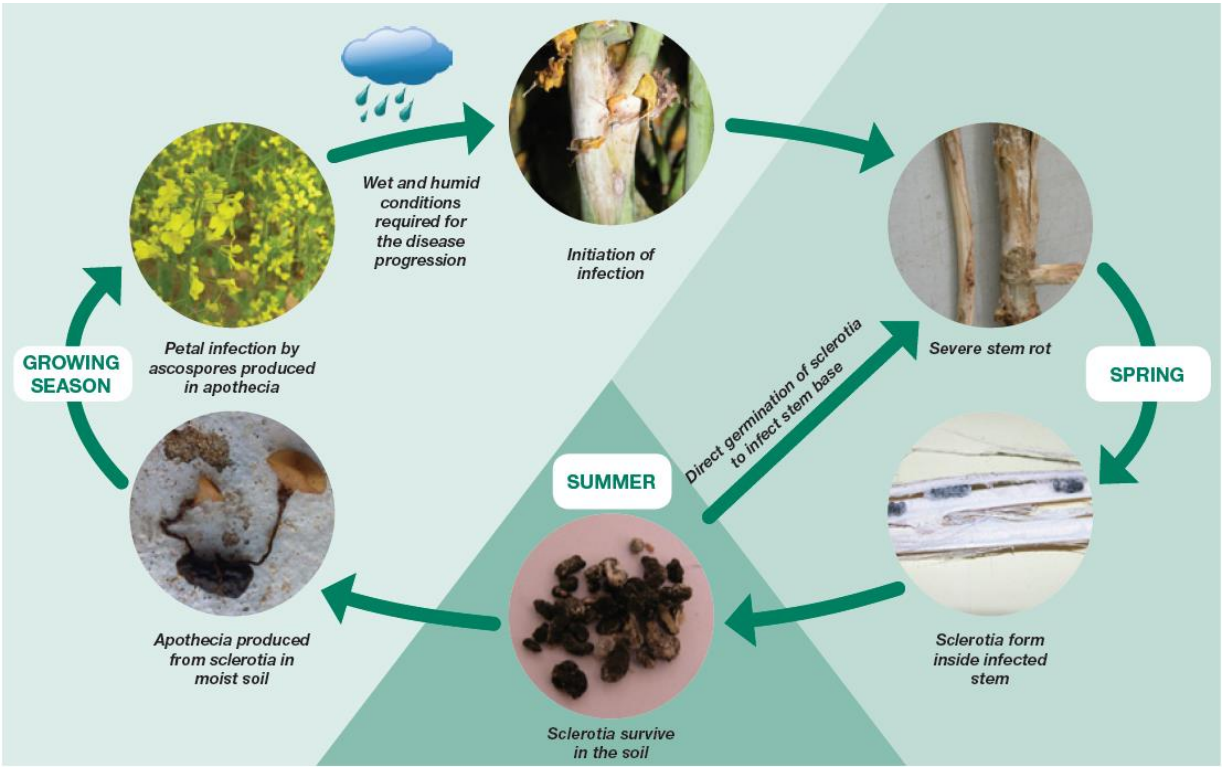


Figure 1.2 Life cycle of *Sclerotinia sclerotiorum* in *Brassica napus.* Reproduced from the Government of Western Australia Department of Agriculture and Food website, https://www.agric.wa.gov.au/canola/managing-sclerotinia-stem-rot-canola.

visible on the leaves and more importantly the stem, giving rise to the common name “sclerotinia stem rot”.

Given the broad host range of *S. sclerotiorum*, crop rotation is not a viable option for disease management. Moreover, a resistant cultivar of canola has not yet been developed due to the complex array of genes that are involved in the infection process. With efforts to find a resistant cultivar and trying to move away from the use of chemical pesticides, more research has been directed towards biocontrol products for managing sclerotinia stem rot in canola.

1.5 Biocontrol

Biological control (biocontrol) is a term that refers to the control of pests by living organisms to benefit the plant host; such organisms are referred to as biocontrol agents. The bacterium *Bacillus thuringiensis* was one of the first biocontrol agents discovered in 1901 for its ability to control caterpillar pests (Bpia 2018). By the 1970’s *B. thuringiensis* was registered as an official biocontrol product across North American (Bpia 2018). During the spore formation process, *B. thuringiensis* releases a protein crystal by-product that elicits an antagonistic effect on larvae (Government of Canada). Currently, there are 14 bacterial and 11 fungal biocontrol agents registered in Canada (Agriculture and Agri-Food Canada 2017).

To be an effective biocontrol agent, the organism must exhibit antagonistic behaviour against the pathogen. Many fluorescent *Pseudomonas spp*. produce antibiotics, volatile compounds such as hydrogen cyanide, lytic enzymes, lipopeptides as well as siderophores (figure 1.3) (Mishra and Arora 2017). These products are produced through secondary metabolism. For most biocontrol bacteria, direct antagonism through antibiosis is the primary mechanism of disease suppression. Moreover, it is quite common that the producer secretes

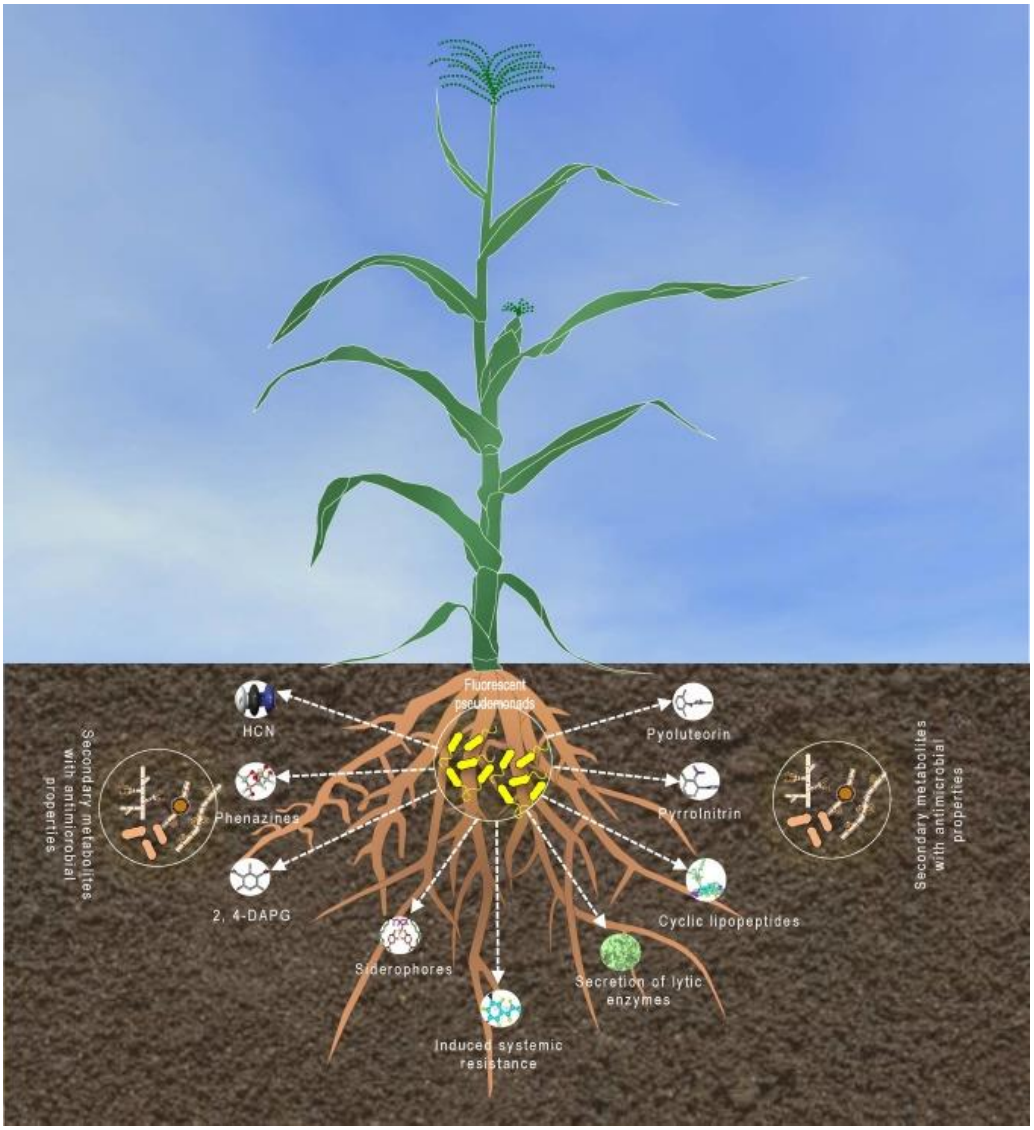


Figure 1.3 Depiction of secondary metabolites produced by fluorescent pseudomonads in soil that have antagonistic characteristics. Reproduced from Mishra and Aurora, Applied Soil Ecology, 2017.

multiple antibiotics that affect different target organisms. Some of the most common antibiotics and their targets are listed below. Cyclic lipopeptides are natural surfactants that function as antibacterial, antifungal or phytotoxic compounds that target cell membranes (Mishra and Arora 2017). Diacetyl phloroglucinol (2,4-DAPG) is a broad spectrum phenolic compound that affects bacteria, fungi and viruses (Mishra and Arora 2017). Phenazines (PHZ) represent a large group of nitrogen-containing heterocyclic ring structures (Mavrodi, Blankenfeldt, and Thomashow 2006). The side groups attached to the ring are responsible for the production of different pigments; for example, pyocyanin is blue in color (Mishra and Arora 2017). PHZs function by forming reactive oxygen species that exhibit toxicity towards cells (Hassan and Fridovich 1980). Hydrogen cyanide (HCN) is produced by many pseudomonads. This organic volatile compound elicits toxicity by interrupting the electron transport chain (Mishra and Arora 2017). Pyrrolnitrin (PRN) is a halogenated aryl pyrrole that interferes with glycerol kinase activity leading to a build-up of glycerol in the membrane causing leakage (Pillonel and Me 1997). This antibiotic is effective against multiple fungal pathogens (Jani et al. 2015).

1.6 *Pseudomonas chlororaphis* strain PA23

First isolated from the roots of soybean plants, *Pseudomonas chlororaphis* strain PA23 has been shown to protect canola against *S. sclerotiorum* in greenhouse and field experiments (Fernando et al. 2007; Savchuk and Fernando 2004; Zhang 2004). Additionally, PA23 can suppress a range of root pathogens, such as *Pythium aphanidermatum, Alternaria solani,* and *Botryodiplodia theobromae* (Kavitha et al. 2005; Savchuk and Fernando 2004). PA23 produces the diffusible antibiotics phenazine-1-carboxylic acid and 2-hydroxyphenazine which give rise to yellow-orange coloured colonies (Zhang et al. 2006). The *phzABCDEFG* gene cluster is

responsible for synthesis of these compounds (Loewen et al. 2014; Selin et al. 2010). Besides PHZs, PA23 produces PRN, which is the product of the *prnABCD* operon (Loewen et al. 2014; Selin et al. 2010). Through mutant generation it was discovered that PRN is the primary antibiotic responsible for PA23 biocontrol of *S. sclerotiorum* (Selin et al. 2010). Interestingly, a PHZ mutant showed increased *S. sclerotiorum* antifungal activity resulting from a 2-fold increase in PRN production (Selin et al. 2010). The reverse was not seen with a PRN mutant; PHZ production remained at levels equal to wild type (wt).

In addition to these antibiotics, PA23 produces the volatile compound HCN. Encoded by the *hcnABC* operon, the HCN synthase is a membrane-associated, oxygen-sensitive enzyme that takes part in cyanogenesis (Devi et al. 2013). HCN antagonizes pathogens by inhibiting the terminal cytochrome c oxidase in the aerobic respiratory chain (Devi and Kothamasi 2009). Other compounds produced by PA23 that may contribute to fungal antagonism include siderosphores, proteases and lipases (de Kievit et al. 2011).

1.7 Regulation

Secondary metabolite production in PA23 is regulated by a complex multi-tiered network that functions at the level of transcription and translation. At the top of this hierarchy is the Gac (global activation of antibiotic and cyanide synthesis) two-component system. A second system is intertwined with Gac called Rsm (regulator of secondary metabolites) (Lapouge et al. 2008). Compiled evidence analyzing diverse groups of bacteria suggests that the Gac/Rsm system is conserved; however, much of what we know about this system is based on the biocontrol strain *P. protegens* CHA0 (Lapouge et al. 2008). Studies have revealed that the sensor kinase GacS initiates a phosphorylation cascade in response to an unknown signal. The phosphate is then

transferred via a phosphorelay system to activate the response regulator GacA (Lapouge et al. 2008). Transcription of small RNAs including RsmX, RsmY and RsmZ is initiated by direct interaction of GacA with an upstream region called the "gac box" (Selin *et al.* 2014; Valverde et al. 2003; Kay et al. 2005; Lenz et al. 2005; Kulkarni et al. 2006). RsmA and RsmE are posttranslational binding proteins that repress translation of target mRNA by binding to and blocking the Shine-Dalgarno sequence (Lapouge et al. 2008). Upon activation of *rsmX, rsmY* and *rsmZ*, the RNAs fold into a flower-like secondary structure with five stem loops that bind the RsmA and RsmE proteins to relieve translational repression (Lapouge et al. 2008).

PA23 also employs quorum sensing (QS) as part of its lifestyle. Thus far, two QS systems have been identified known as PhzRI and CsaRI; however, only the Phz system has been studied in detail. The *phzI* gene encodes an AHL synthase that generates acyl-homoserine lactones (AHL) that function as inter-cellular signalling molecules (Selin, Fernando, and de Kievit 2012). As the cell population increases, AHL concentrations reach a threshold level, which permits AHL binding to the transcriptional activator PhzR (de Kievit et al. 2011). The activated PhzR complex binds to a "phz box" located upstream from the *phz* operon and initiates transcription of the PHZ biosynthetic genes (Selin et al. 2012). PRN, protease and biofilm production have also been shown to be under QS control (Selin et al. 2012).

Additional regulators that influence secondary metabolite production include the stationary sigma factor RpoS. RpoS functions in response to environmental changes, such as nutrient availability, osmotic stress and temperature shock (Hengge-Aronis, 2002). In PA23, RpoS negatively affects biocontrol as an *rpoS* mutant exhibits higher antifungal activity (Manuel et al. 2012). PRN and protease production are negatively regulated by RpoS, so a mutation in *rpoS* results in elevated levels of these compounds leading to increased fungal suppression (Manuel et

al. 2012). RpoS is positively regulated by both QS and a transcriptional regulator called PsrA (Selin et al. 2014). The stringent response is a global stress response that also has ties to *rpoS*. During nutrient deprivation, the stringent response enables bacteria to shift from a reproductive state to a survival mode. In PA23, the stringent response negatively affects PRN, lipase and protease activity by upregulating *rpoS* expression; thus, stringent response mutants exhibit enhanced fungal suppression (Manuel et al. 2012).

An anaerobic regulator, called ANR, has been recently identified that affects secondary metabolite production in PA23. ANR was found to positively regulate HCN, PRN, PHZ and protease production and so not surprisingly, *anr* mutants show no *S. sclerotiorium* suppression (Nandi et al. 2016). In addition, ANR activates *phzI* and *phzR* while PhzR represses *anr* expression (Nandi et al. 2016). In *Pseudomonas aeruginosa*, ANR and QS show extensive cross regulation with each other similar to what is observed in PA23 (Hammond *et al*., 2015; Schuster & Greenberg, 2006).

1.8 Plant growth promotion

Many bacteria with antagonistic behaviour against pathogens may also benefit plant growth directly. Plant growth promoting rhizobacteria (PGPR) is a term that refers to bacteria that initiate a positive relationship with host plants that stimulates growth. Plants have their own complex immune defense systems that detect pathogens and ultimately affect plant growth. Ethylene is a stress hormone that is synthesized during pathogen attack or upon exposure to changes in temperature, nutrients or light (Glick 2012). This simple hormone is active at low concentrations and affects plant development (Glick 2012). Ethylene can inhibit root elongation, promote pre-mature fruit ripening and activate synthesis of hormones that prevent root nodule

formation by *Rhizobia* spp (Glick 2012). Some PGPR produce 1-aminocyclopropane-1-carboxylated (ACC) deaminase that degrades ACC, a precursor for ethylene production, thereby reducing the plant stress response. It has been reported that PA23 does not produce ACC deaminase (Reimer 2016).

Phytostimulator is a term given to some PGPR, referring to their ability to synthesize and secrete plant hormones that promote plant growth directly (Pérez-Montaño et al. 2014). Indoleacetic acid, IAA, is an auxin naturally produced by plants to stimulate growth and development (Glick 2012). IAA can be synthesized by some PGPR to encourage growth and establish a beneficial relationship with the plant (Glick 2012). A study with *Pseudomonas putida* GR12-2 showed that IAA encouraged longer root growth compared to plants treated with an IAA-deficient strain (Patten and Glick 2002). PA23 has been found to produce IAA (Reimer 2016). Other hormones produced by PGPR include cytokinins and gibberellins. In 2008, a study conducted with Arabidopsis showed the cytokinins produced by *Bacillus megaterium* increased root and shoot growth (Ortíz-Castro, Valencia-Cantero, and López-Bucio 2008). Gibberellins have been implicated in an array of plant developmental processes, such as seed germination, emergence, maturation, root and vegetative growth (Bottini, Cassán, and Piccoli 2004).

Nutrient acquisition is another means by which PGPR can influence plant growth. Nitrogen is often a limited nutrient in soil environments and not present in a usable form for plants. Some PGPR possess the ability to fix nitrogen for the host plant by converting nitrogen (N_2) to ammonia (Pérez-Montaño et al. 2014). Phosphorus is also required for plant cell growth and metabolism. Despite its relative abundance in soil, phosphorus is predominantly in an insoluble form (Pérez-Montaño et al. 2014). PGPR solubilize phosphorus into a usable form that plants can absorb (Pérez-Montaño et al. 2014). Iron is an essential metal that is present in soil as

Ferric iron (Fe^{+}), which is inaccessible to plants (Glick 2012). Siderophores are molecules produced by some bacteria that bind and convert Fe^{+} to a soluble form (Glick 2012). Iron acquisition can also benefit the plant indirectly by starving phytopathogens of this essential element.

1.9 Volatile organic compounds

More recently, volatile organic compounds have been implicated in PGP through their role as signals. PGPR can produce an array of different volatile compounds including aliphatic aldehydes, esters, alcohols, organic acids, sulfur compounds and ketones (Vaishnav et al. 2017). *Arabidopsis thaliana* seedlings showed increased root and shoot mass when exposed to volatiles from four different *Bacillus* strains (Asari et al. 2016). In another study, an increase in biomass was observed in tobacco (*Nicotiana tabacum*) plants in the presence of *Pseudomonas fluorescens* strain ss101 volatiles (Park et al. 2015). Gas chromatography and mass spectrometry revealed 3 main volatiles were being produced, each with different plant promotion effects (Park et al. 2015).

PA23 produces volatiles with the major compounds being 2-ethyl-1-hexanol, 2-nonanal and 3-benzothiazole (Fernando et al. 2005). These molecules have been shown to inhibit *S. sclerotiorum* mycelial growth and formation of sclerotia bodies (Fernando et al. 2005). The PGP capabilities of the aforementioned volatiles have not been explored, and are something that requires further investigation.

1.10 Marketing biocontrol agents

For over a decade, research has demonstrated that PGPR can effectively suppress disease in both laboratory and field experiments. However, there are challenges that need to be met to market a product that farmers can use effectively (Bashan et al. 2014). First, the biocontrol product needs to be easy to use. Elaborate preparation procedures will be costly and overwhelming for the consumer. For example, if cell cultures require storage at -80 degrees Celsius, many farmers do not have the equipment necessary for maintaining that temperature. Second, the microorganisms must remain viable for long periods of time using basic storage methods (Bashan et al. 2014). This can be accomplished by creating a formulation that introduces additives that improve cell survival and retain biocontrol properties (Bashan et al. 2014). For example, a liquid formulation containing glycerol was found to maintain *P. fluorescens* Pf1 viability for 6 months (Manikandan et al. 2010). Glycerol can protect cells from desiccation because of its water holding potential which slows the rate of drying (Manikandan et al. 2010). It has been suggested that a good formulation should be able to maintain cell viability for at least one year upon storage at either room temperature or 4^{o}C (Bashan et al. 2014). In addition, the manufacturing cost and complexity of the biocontrol product must remain as low as possible (Bashan et al. 2014). If the biocontrol preparation to too expensive, farmers will be deterred from using such products. Lastly, it is important that formulations contain additives that are environmentally friendly and safe for humans (Bashan et al. 2014).

In addition to various additives that support cell survival, different strategies exist for preparing cell formulations. Figure 1.4 displays commonly used formulations for PGPR (Bashan et al. 2014). Inorganic, organic and polymeric inoculants are primarily used for soil and seed treatment, while both liquid and dry formulations are employed for plant, soil or seed

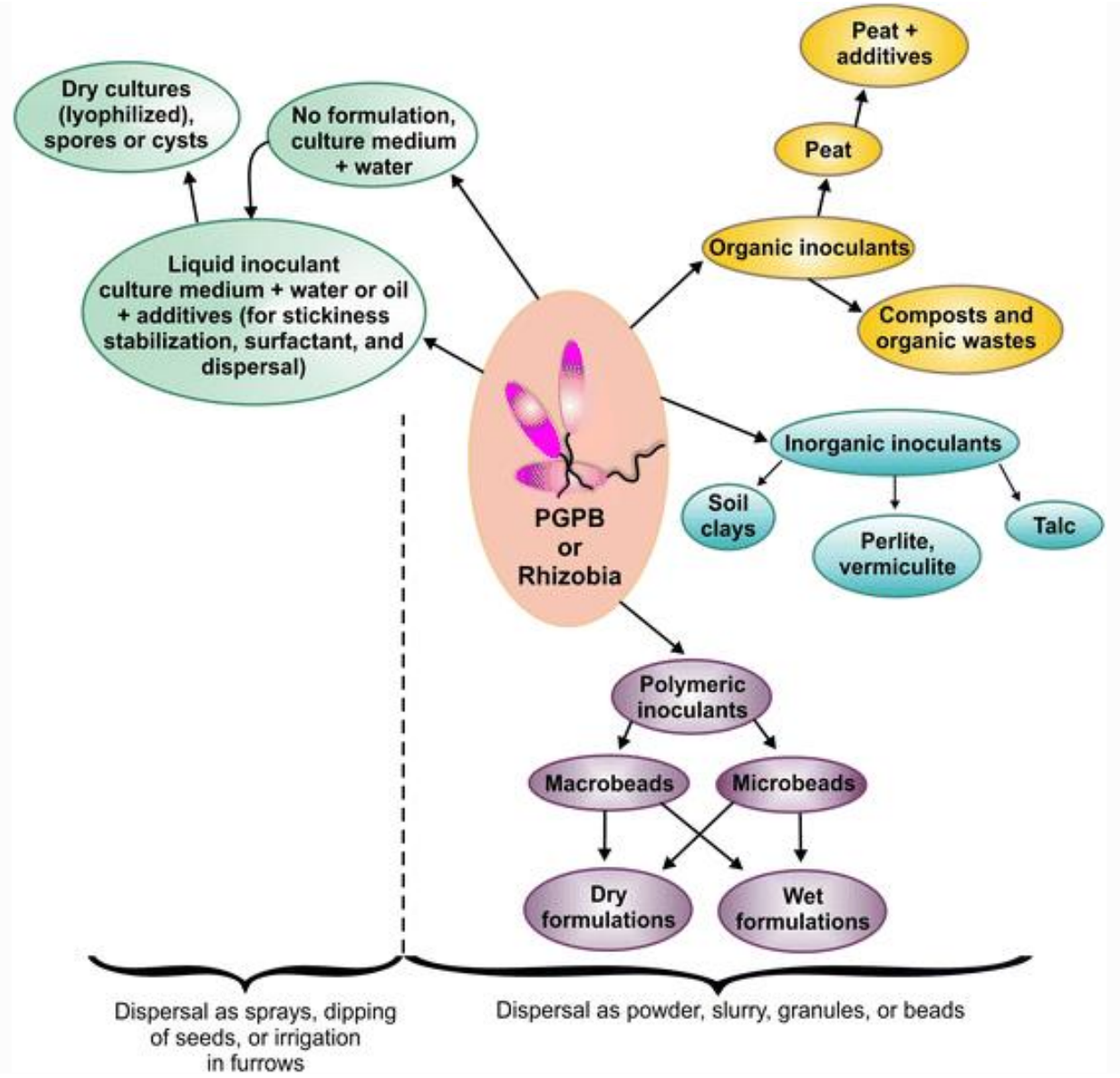


Figure 1.4 Depiction of various inoculant formulations for different application methods. Reproduced from Bashan et al., Plant and Soil, 2014.

applications (Bashan et al. 2014). Unlike gram-negative bacteria, gram positive bacteria produce spores that can be easily formulated using most of the methods shown in figure 1.4 due to their resiliency. Conversely, selecting a formulation for gram-negative bacteria can be a challenge. It is important to select an application method prior to choosing a suitable formulation. For example, *S. sclerotiorum* causes disease in the phyllosphere and so, in this case, a spray or liquid application method would be most suitable. A liquid or dry inoculant formulation would be preferable for a spray or liquid culture application. Lastly, to successfully market a biocontrol formulation, it must provide reproducible results in farmers' fields under varying conditions. Despite these challenges, more research and commercial products are emerging that incorporate PGPR to prevent disease and promote plant growth.

1.11 Thesis objectives.

P. chlororaphis strain PA23 is a biocontrol agent that is able to protect against fungal diseases that threaten canola. At present, there is very little known about the PGP effects associated with diffusible or volatile compounds produced by PA23. Moreover, an effective storage formulation for PA23 has not been developed. To address these knowledge gaps the objectives of this thesis are as follows:

1. To observe the PGP abilities of PA23 on canola and lettuce when applied via drench irrigation.
2. To investigate changes in biomass of lettuce seedlings when exposed to organic volatile compounds produced by PA23 in an enclosed system.
3. To evaluate the shelf life of various storage formulations containing PA23.

2. METHODS AND MATERIALS

2.1 Bacterial growth conditions

Pseudomonas chlororaphis PA23 and derivative strains were routinely cultured at 28^{o}C on Lysogeny (LB) media (Difco Laboratories, Detroit, MI, USA). For long-term storage, bacteria were maintained as skim milk (10%) stock cultures stored at -80^{o}C.

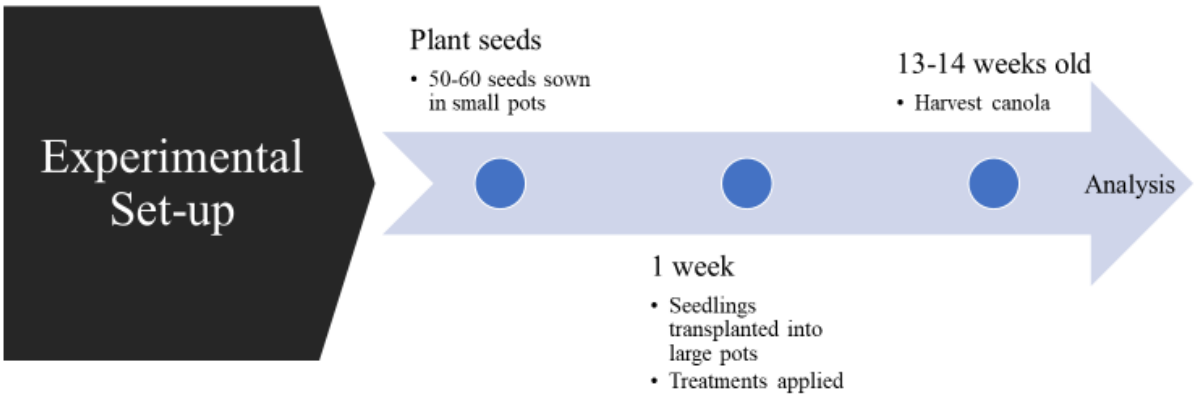
2.2 PGP assays in soil

Plants were grown in growth chambers set to 21^{o}C, 16h light/8h dark and 0% humidity. Plants were watered every second day.

2.2.1 Mature canola

Figure 2.2.1A, depicts the experimental design of the first set of PGP assays (top panel I) and the bacterial treatments employed (bottom panel II). A total of 60 canola seeds (Fernando lab, University of Manitoba) were sown in small pots (14x11x6.2 cm) containing moistened soil that was packed down and filled to the top, then placed in the growth chamber. After one week of growth, a total of 42 seedlings were transplanted into large round pots (20x18 cm), one plant per pot. Overnight cultures of the PA23 wt and the *gacS* mutant (1L volume in LB) were pelleted and cells were re-suspended in fresh LB at the following concentrations: $2x10^{7}$cfu/ml (OD_{600} = 0.01), $2x10^{8}$cfu/ml (OD_{600}= 0.1) and $2x10^{9}$cfu/ml (OD_{600} = 1.0). Seedlings were drenched with one of the six bacterial treatments or LB broth for the control; 6 plants were included per treatment (figure 2.2.1A, diagram II). After 13 weeks in the growth chamber, plants were harvested, the roots were washed to remove adherent soil and then subject to biomass analysis.

I.



II.

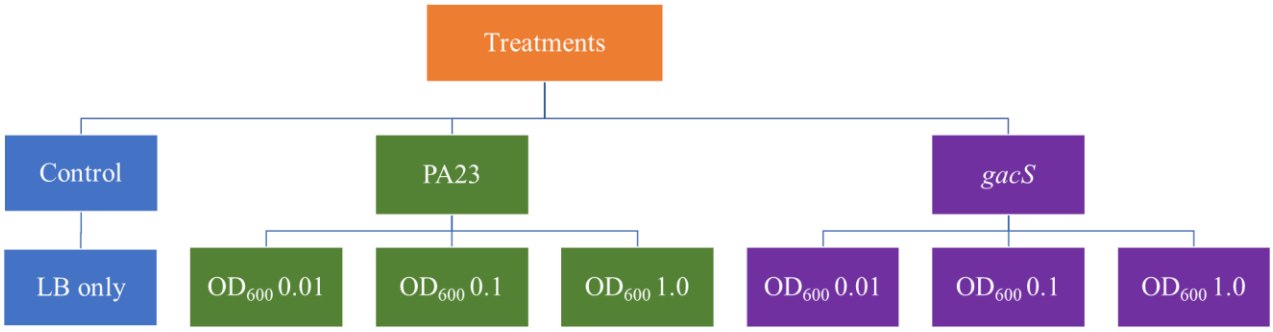


Figure 2.2.1A Experimental set-up for testing the PGP effects of the wild-type PA23 and the *gacS* mutant (top panel I) and the various bacterial treatments applied (bottom panel II). n=6

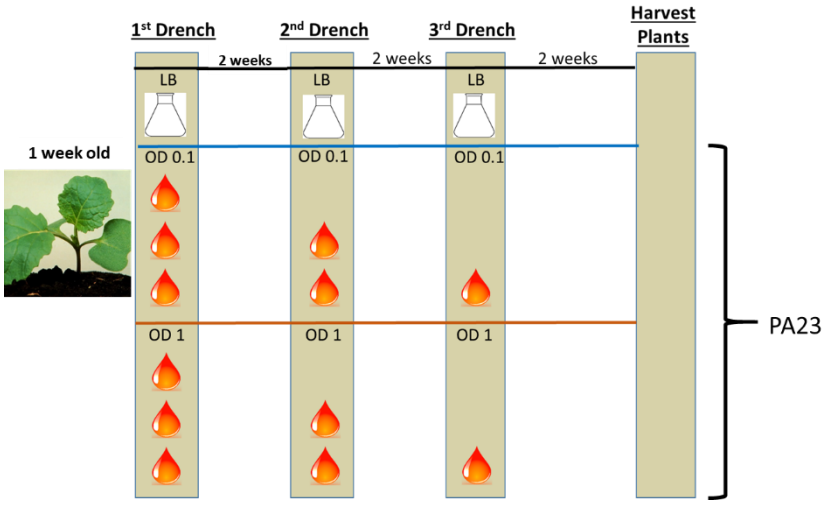
The next experiment with canola was executed in a similar manner; however, the treatment groups were changed. Figure 2.2.1B, outlines the experimental set up employed for trials 2 and 3. In trial 2, the impact of multiple PA23 drenches at an OD_{600} of 0.1 and 1.0 was investigated (top panel I). Trial 3 was similar to trial 2 except that bacterial treatments included both PA23 and the *gacS* mutant at an OD_{600} of 1.0 (bottom panel II). Overnight cultures of PA23 and the *gacS* mutant were spun down, pelleted and standardized to OD_{600}=1.0 with fresh LB. After 13 weeks of growth plants were harvested and biomass was analyzed.

2.2.2 Canola seedlings

In the next set of experiments, canola plants were harvested at an earlier time point to determine the impact of PA23 on earlier stages of plant development. Canola seedlings that were harvested 7 days post treatment (dpt), seeds were sown in small pots (11x14x6.2 cm) with moistened soil. A total of 56 seeds were sown, four seeds per pot, 2 pots per treatment. After one week of growth, 25ml of culture was applied by drench irrigation. Overnight 600ml cultures (16h) of PA23 and the *gacS* mutant were pelleted and re-suspended in fresh LB broth to standardize cultures to the following concentrations: $2x10^7$cfu/ml; $2x10^8$cfu/ml; $2x10^9$cfu/ml. Seven days after the irrigation treatment was applied, plants were harvested and analyzed (figure 2.2.2). Experiments were repeated three times.

Seedlings that were harvested at 14 and 21 dpt, seeds were sown in medium sized pots (15x13 cm) with moist soil (3 seeds per pot). Bacteria were prepared as described above and a 50ml volume was applied to 7-day old plants. Plants were harvested 2 and 3 weeks later (figure 2.2.2) and subject to biomass analysis. Experiments were repeated three times.

I.



II.

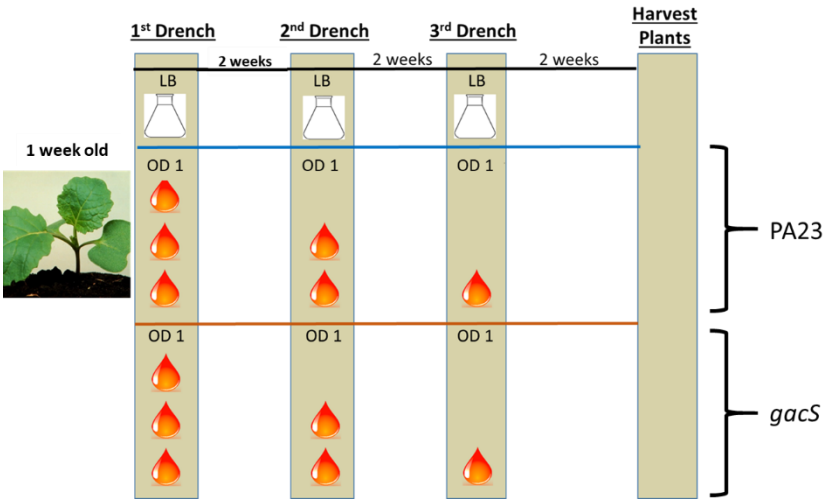


Figure 2.2.1B Treatment set-up for trials 2 (top panel I) and 3 (bottom panel II) investigating PGP by PA23. n=6

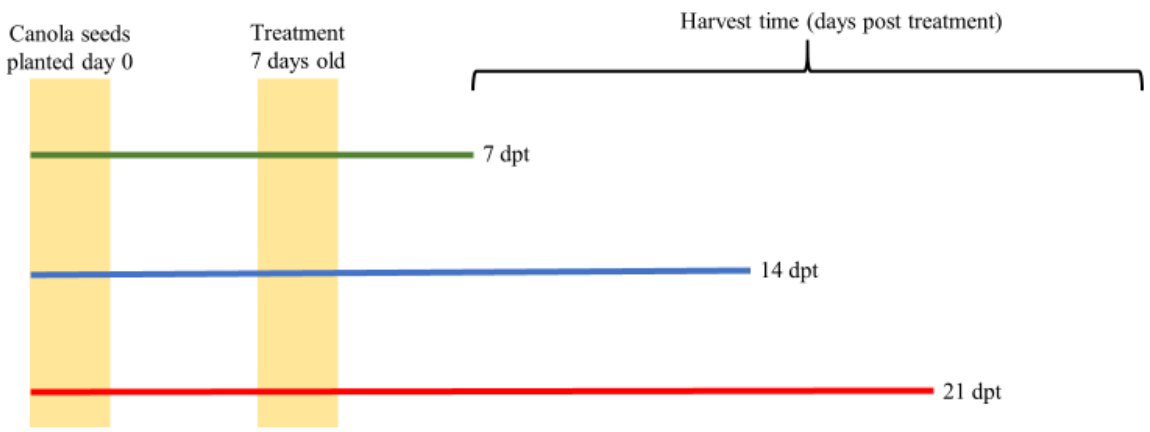


Figure 2.2.2 Diagram of treatment timeline and time of harvest. n=8 (7 dpt), n=6 (14 dpt and 21 dpt).

2.2.3 Lettuce

Lettuce seeds (Heritage, Brandon, MB, CA) were sown into 10 small pots with moistened soil, 5 seeds per pot and placed in the growth chamber. After 3 weeks of growth, lettuce seedlings were transplanted into larger pots with moistened soil; a total of 42 plants were transplanted, 2 plants per pot, 3 pots per treatment. Overnight cultures (600ml) of PA23 and the *gacS* mutant were pelleted and resuspended in fresh LB at the following concentrations: $2x10^7$cfu/ml; $2x10^8$cfu/ml; $2x10^9$cfu/ml. Transplanted lettuce seedlings were drenched with 50ml of the aforementioned bacterial preparations or LB broth (no bacteria control) and placed in the growth chamber (figure 2.2.3). After two weeks of growth lettuce plants were harvested. Experiments were repeated three times.

2.3 Biomass analysis

For both the canola and lettuce studies, plant biomass was analyzed by determining root/shoot length and fresh/dry weight. In addition, the weight of canola seeds harvested from mature Westar plants was monitored.

2.4 Enclosed bi-plate assay for volatiles

Bi-plates were created such that one half of the plate contained 20ml of 0.3% phytagel (Sigma-Aldrich, Darmstadt, Germany) and 0.22% Murashige and Skoog agar media (MS) (Sigma-Aldrich) amended with sucrose (2%) (pH5.8). The other side of the plate contained 10ml LB agar. Lettuce seeds were sterilized by rinsing two times in 50% ethanol for 30 seconds followed by a rinse with sterile water and soaking in a 1% bleach solution for 15 minutes. The

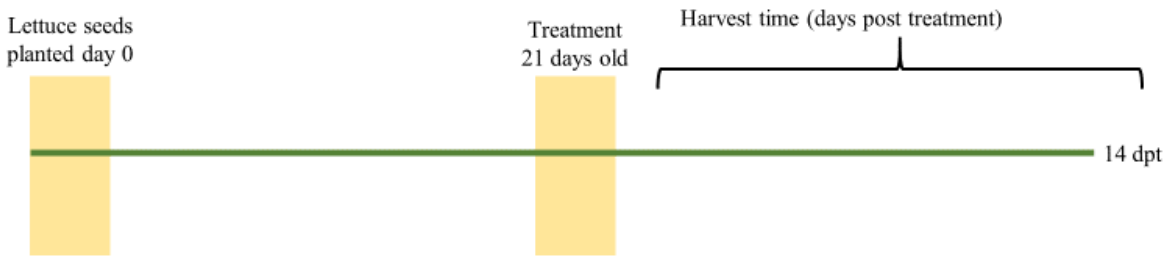


Figure 2.2.3 Treatment timeline for lettuce PGP assays. n=6

seeds were then washed thoroughly with sterile water. Using sterilized forceps, lettuce seeds were placed on the MS agar, the plates were sealed with parafilm and grown under fluorescent T5 lights (kleengro, Wpg, MB, CA). Plants were allowed to grow for one week before bacteria were inoculated onto the second half of the plate. Overnight cultures (3ml) of the PA23 wt, and the *gacS* and *hcn* mutants standardized to an OD_{600}=0.1 were inoculated onto the LB side of the plate as a 10µl volume. The bi-plates without lids were placed in sterilized glass jars (11x12 cm) and the lids were wrapped with parafilm. The plates were open-faced inside the jars to allow space for the plants to grow in an enclosed environment containing bacterial volatiles. The lettuce seedlings were grown in jars for two weeks at room temperature under T5 fluorescent lights set to 16h light/8h dark. The experiment was repeated twice. Figure 2.4 shows the experimental set-up in the jars.

2.5 Preparation of PA23-based formulations

2.5.1 Liquid formulation preparation

Cultures of PA23 (2000ml) grown for 16 hours in LB broth were pelleted and re-suspended with LB broth plus the following additives: corn starch (3.8%); polyethylene glycol (PEG, 0.1%); silwet (0.2%); corn steep liquor (CSL, 0.1%); combo #1 (corn starch, PEG and CSL); combo #2 (corn starch, PEG and silwet) and water (control). See table 2.5.1 for a summary of all the additives used in the liquid formulations. Re-suspended cultures were concentrated to achieve a target concentration of $1x10^{10}$ cfu/ml (10x concentrated). For each treatment 20ml aliquots of PA23 formulated sample was added into 10 mylar screw cap foil bags (Sorbent Systems, Los Angeles, CA, USA) and stored at 4^{o}C for 12 months (figure 2.5.1).

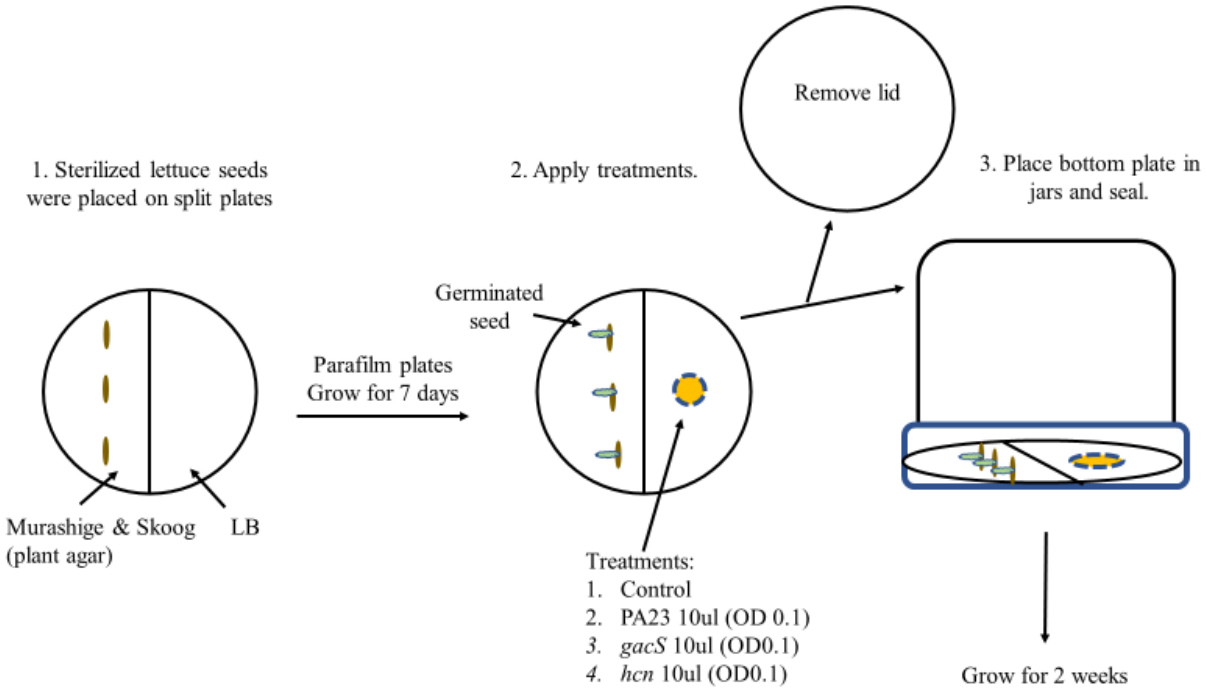


Figure 2.4 Flow diagram of enclosed volatile bi-plate assay. n=12-15.

Table 2.5.1 Description of additives for liquid formulations

Media abbreviation	**Additive concentration (%)**
Lysogeny broth (LB)	-
LB + Corn starch (CS)	3.8%
LB + Polyethylene glycol (PEG)	0.1%
LB + Silwet	0.2%
LB + Corn steep liquor (CSL)	0.1%
LB + Combo #1	CS (3.8%), CSL (0.1%) and PEG (0.1%)
LB + Combo #2	CS (3.8%), Silwet (0.2%) and PEG (0.1%)

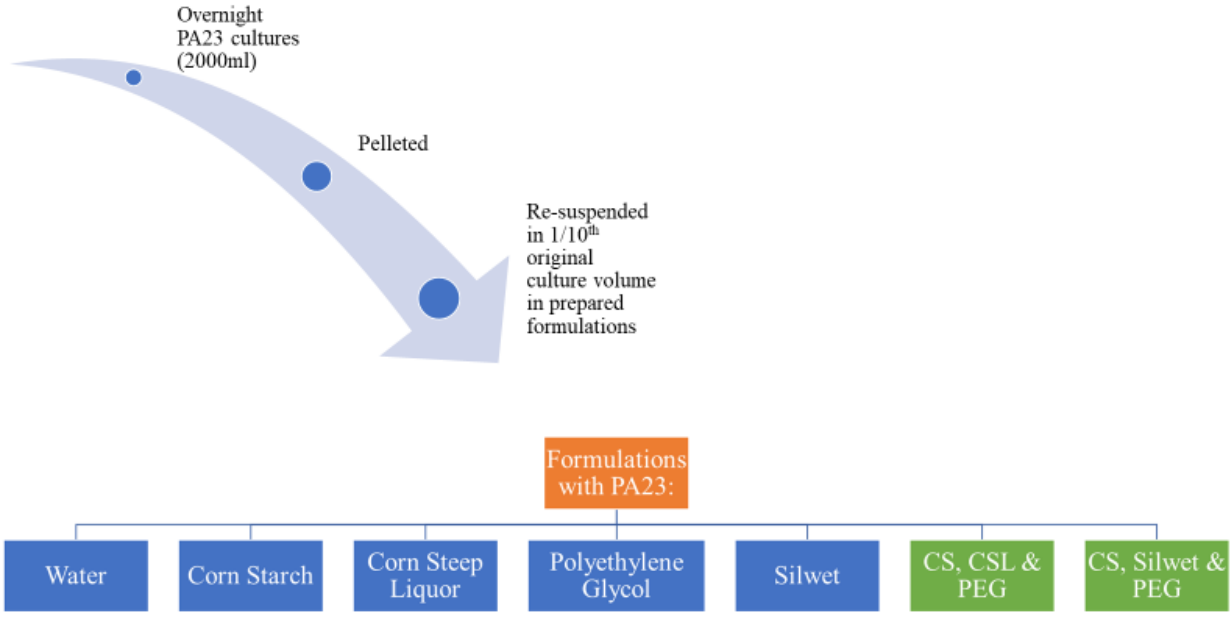


Figure 2.5.1 Liquid formulation workflow depicting culture preparation and formulation strategy.

Repeated exposure to air and room temperatures by repeated sampling from the same bag may decrease viability quicker. Therefore, a new bag was opened every time the cell viability was tested for each sample to ensure that each bag was opened once.

2.5.2 Dry formulation preparation

For the first set of samples, PA23 was grown in 300ml of LB broth (standard culture) for 16h and 600ml of M9 minimal salts medium [amended with 0.2% glucose, 1mM magnesium sulfate ($MgSO_4$), 0.3M NaCl and 0.1mM glycine betaine (GB)], denoted as $M9^+$, for 24h. Table 2.5.2 defines the media and abbreviations used for the dry formulations. Figure 2.5.2 shows a workflow of the dry formulation experiments and the treatment groups. Cultures were pelleted, and re-suspended in M9 media (no glucose and $MgSO_4$) with lactose (150g/L). PA23 cultures that were grown in LB broth were concentrated 10x, $M9^+$ cultures with PA23 were concentrated 20x due to the lower number of cells. A concentration between $1x10^{10}$ – $1x10^{11}$ cfu/ml prior to lyophilization was the target to account for loss of cell viability during the freeze-drying process. A volume of 10ml of each formulated sample was transferred into cryo-glass vials (Fisher Scientific) and three biological replicates were created for each sample formulation. After transferring into cryo-glass vials, the tops were covered with parafilm and then cheesecloth and secured with an elastic band prior to freezing overnight at -70^{o}C. The next day, frozen samples were placed on the freeze dryer (1.4Pa at -51^{o}C) for 5-7 days. After samples were completely dry, the cheesecloth and parafilm were removed and vials were covered with twist cap lids. Samples were stored in the fridge at 4^{o}C for 12 months.

The second set of samples were grown in 300ml LB broth (8h), 600ml M9 media (10h), 900ml $M9^+$ media (16h) and 300ml TB broth (7h) to collect cultures at the beginning of

Table 2.5.2 Media description and additives used for dry formulations.

Media Abbreviation	Description
LB	Lysogeny broth
M9	M9 minimal salts media with 0.2% glucose + 1mM $MgSO_4$
$M9^+$	M9 minimal salts media with 0.2% glucose, 1mM $MgSO_4$, 0.3M NaCl and 0.1mM glycine betaine
TB	Terrific broth (1.2% tryptone, 2.4% yeast, 0.4% glycerol, 0.017M KH_2PO_4 and 0.072M K_2HPO_4)
Lactose	150g/L

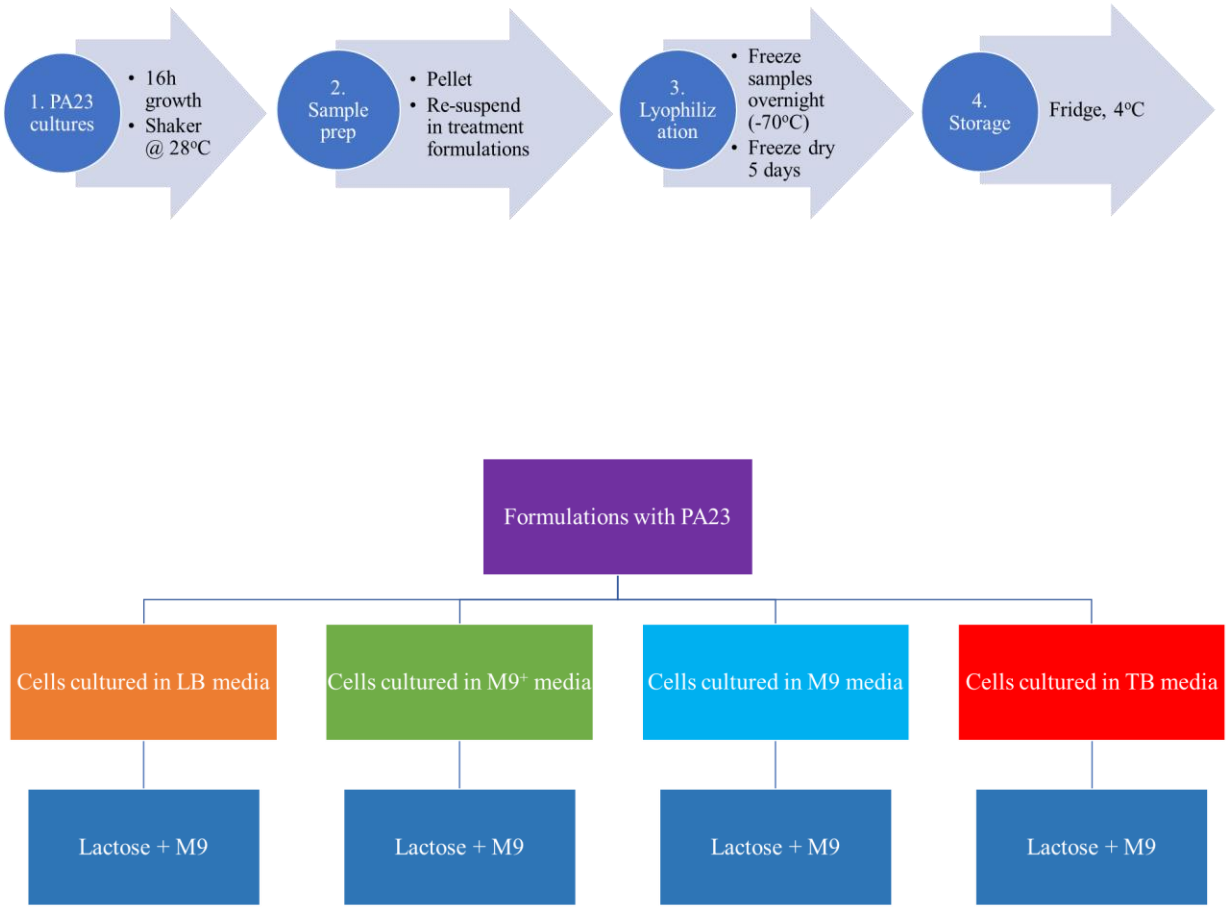


Figure 2.5.2 Workflow for the sample preparation for dry formulations.

stationary phase. Samples were pelleted and re-suspended in M9 with no additives except lactose (150g/L). Because a higher loss of viability due to freeze-drying was observed with the first set of samples, cells were concentrated to between $1x10^{11}$- $1x10^{12}$ cfu/ml. To achieve this cell number, LB and TB cultures were concentrated 20x, M9 cultures were concentrated 40x and $M9^{+}$ cultures were concentrated 60x. Cells were re-suspended and a 5ml volume was transferred into each cryo-vial; three biological replicates per sample formulation were created. Glass cryo-vials were covered with cheesecloth and parafilm and then tied with an elastic band and frozen overnight at -70^{o}C. The next morning, samples were placed on the lyophilizer (same conditions as set 1) and dried for 4 days. The drying took less time because the volume was decreased from 10ml to 5ml in the second experiment.

2.6 Viability analysis

To determine the viability of the PA23 liquid and dry formulations, samples were taken at various time points during storage for CFU determination (see figure 2.6). The concentration of cells in fresh cultures prior to lyophilization was tested by measuring the OD_{600}. Immediately after lyophilization and at various time points during storage, CFU counts were taken to determine viability. A 1ml volume of liquid formulation was added to 9ml H_2O; serial dilutions were generated and then spread plated onto LB agar. After 48h growth at 28^{o}C, colonies were counted. Dry formulations were handled in a similar manner except 0.01g of sample was first dissolved in 1ml M9 (no glucose or $MgSO_4$) to re-constitute cells. The reconstituted samples were diluted in series, transferring 1ml into 9ml and a 100ul volume was spread onto LB agar plates. All plates were incubated for 2 days at 28^{o}C.

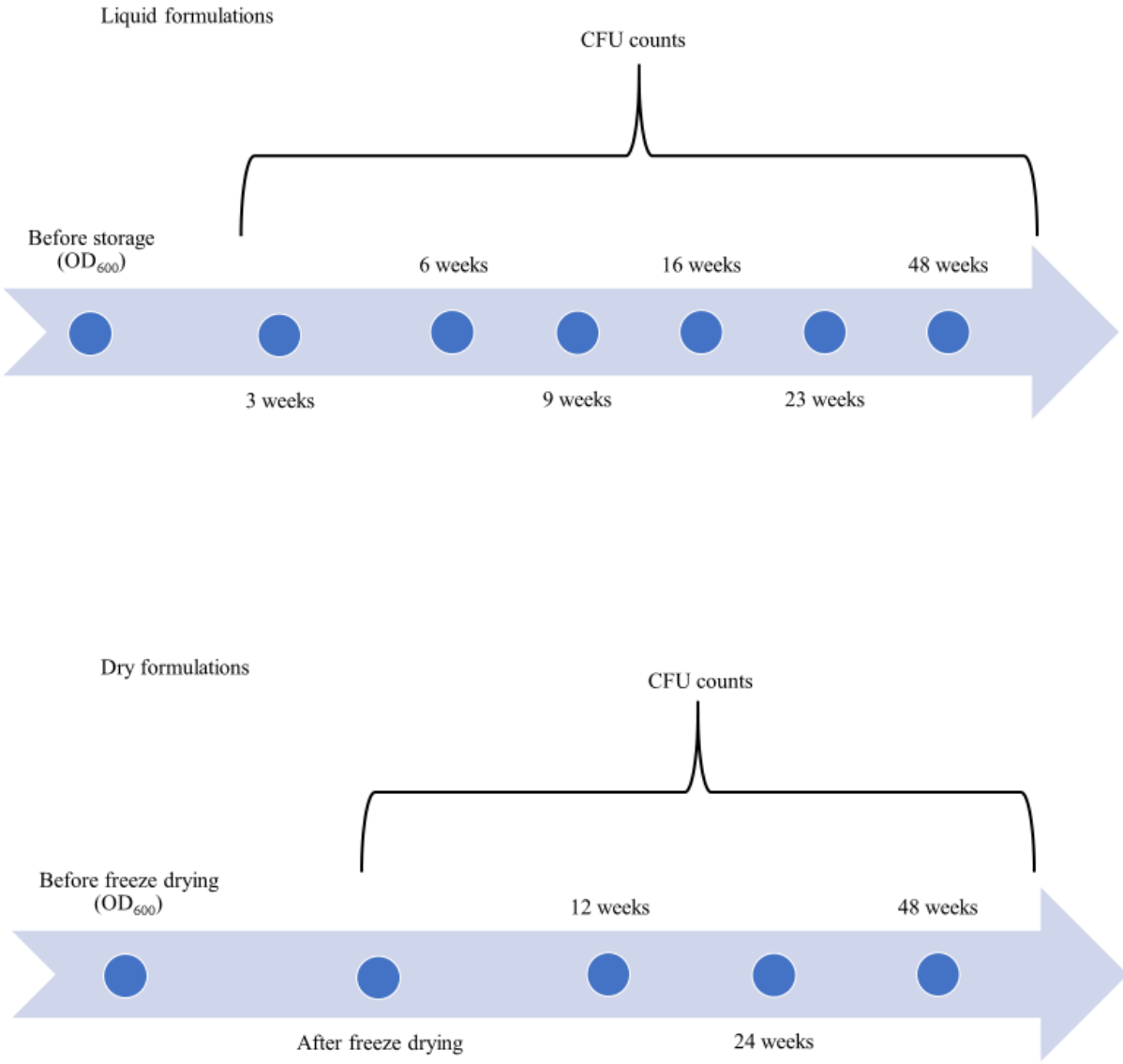


Figure 2.6 Timeline of formulation viability testing for liquid and dry formulations.

2.7 Phenotypic assays

Cells from the liquid and dry formulations were analyzed to determine if they were phenotypically similar to the PA23 wt. Ten isolated colonies from each sample were streaked onto an LB agar plate and incubated overnight at 28^{o}C. A single colony was used to inoculate LB broth and cultures were grown for 16h at 28^{o}C. Cultures were standardized to a concentration of $2x10^{9}$ cfu/ml and a 5µl volume was used to inoculate the phenotypic assay plates described below.

2.7.1 Antifungal activity

To observe the antifungal activity of the liquid and dry formulations compared to fresh PA23, radial diffusion assays were performed. A 5µl volume of bacterial preparations described above were spotted onto 1/5 strength PDA and grown at 28^{o}C for 48h. After which, an agar plug containing fungal mycelia was placed in the center of the plate. Plates were stored for 4 days at room temperature to allow *S. sclerotiorum* to grow. Inhibition of fungal growth was assessed by measuring the zone of clearing between the edge of the bacterial colony and the fungal mycelia (Poritsanos et al. 2006).

2.7.2 Protease

Protease activity was observed by inoculating 5µl from overnight cultures onto bacto-agar plates (1.5%) containing skim milk (2%). Proteolysis surrounding the bacterial colonies was apparent after 36-48h of growth at 28^{o}C (Poritsanos et al. 2006). The zones of clearing were measured at 48h and 72h.

2.7.3 Autoinducer

Production of AHL molecules was analyzed qualitatively by spotting 5µl from overnight cultures onto LB agar plates seeded with *Chromobacterium violaceum* CVO26. This strain is AI-deficient and can be used to detect exogenous production of AHLs with a carbon chain length ranging from C_4 to C_8. When exposed to exogenous AHLs, a purple halo (violacein) will be present around bacterial colonies (Latifi et al. 1995). The diameter of the purple halo was measured at 48h.

2.8 Infection assay

To assess the biocontrol capability of the liquid and dry PA23 formulations, canola plants were challenged with *Leptosphaeria maculans* in the presence and absence of bacteria. Wounds were made on the cotyledons of two-week-old Westar seedlings via curled forceps. Liquid formulations were spun down, pelleted and re-suspended in fresh LB and the dry formulations were re-constituted by mixing with fresh LB broth. Immediately after sample preparation, the cotyledon wounds were inoculated with 10µl of the PA23 formulated samples standardized to a concentration of $8x10^8$ - $1x10^9$ cfu/ml. A 10µl volume of *L. maculans* spore suspension in water ($5x10^6$ spores/ml) was used to infect each cotyledon. Control plants were treated with LB broth only (healthy control), or with pathogen only (disease control). Seedlings were grown in the growth chamber for 2 weeks, after which fungal lesions were measured.

3. RESULTS

3.1 PGP was variable in mature canola.

In order to investigate the impact of PA23 on PGP, 7-day old canola seedlings were irrigated with either the PA23 wt or the *gacS* mutant. In addition, various concentrations of bacteria were applied (figure 2.3.1A). After 13 weeks of growth, canola plants were harvested and analyzed. Figure 3.1.1 shows selected plants from each of the seven treatment groups. Visually, plants that were treated with PA23 cultures adjusted to an OD_{600}=0.1 ($2x10^8$cfu/ml) and OD_{600}=1.0 ($2x10^9$cfu/ml) appeared larger than the other treatment groups. Figure 3.1.2 displays the average plant measurements of fresh/dry weight and root/shoot length. At all three concentrations PA23 treated plants showed a higher fresh and dry weight compared to control plants. A significant increase in fresh weight was seen in plants that were treated with the *gacS* mutant OD_{600} =1.0 ($2x10^9$cfu/ml) cultures; however, after drying there was no significance observed. Only plants treated with the *gacS* mutant OD_{600} =0.01 ($2x10^7$cfu/ml) had a significantly higher dry weight compared to controls plants. None of the treatments resulted in increased shoot length. In terms of root length, plants treated with PA23 (all concentrations) and the *gacS* mutant (OD_{600} =0.1 and OD_{600} =1.0) had longer roots compared to control plants.

For the most part, the greatest increase in plant biomass resulted from PA23 irrigation at the higher concentrations (OD_{600}=0.1 and OD_{600}=1.0); therefore, the next set of plant trials was designed to test the effect of multiple doses of PA23 at these two concentrations. As outlined in Figure 2.3.1B, canola plants were treated with one, two or three doses of PA23. Figure 3.1.3 shows the 13-week old mature canola plants prior to harvest. Plants drenched multiple times with PA23 at either concentration showed no obvious difference upon visual observation**.** Graphical

Figure 3.1.1 Picture of 13-week old canola plants before harvesting for biomass analysis. Three different concentrations of PA23 and the *gacS* mutant (OD_{600} =0.01, $2x10^7$; OD_{600} =0.1= $2x10^8$; OD_{600} =1.0= $2x10^9$cfu/ml) were used for plant drenching. n=6.

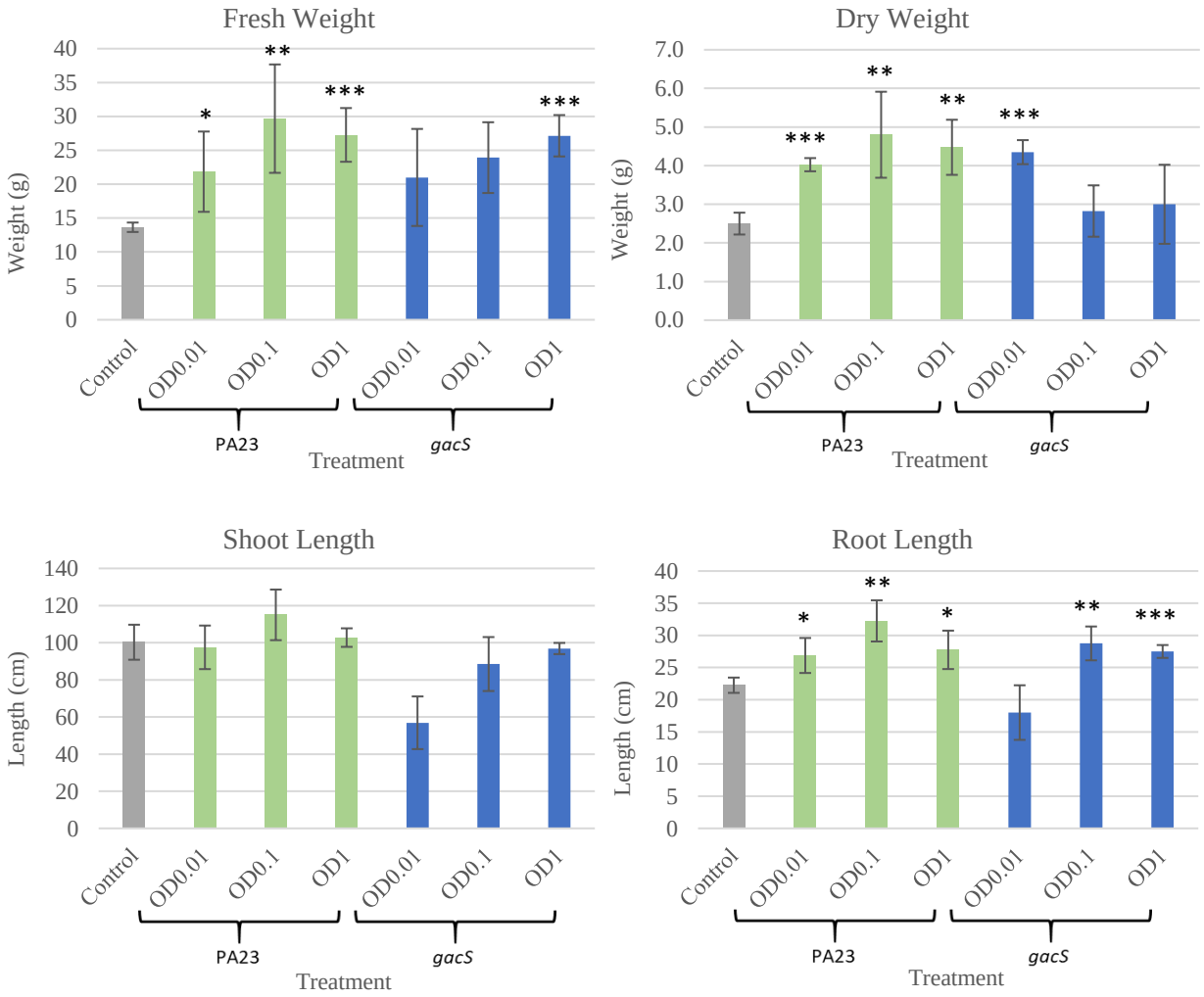


Figure 3.1.2 The effect of the PA23 wt and the *gacS* mutant on canola biomass, shoot and root development. Plants were treated with three different concentrations of bacteria and analyzed after 13 weeks of growth. (OD_{600} =0.01, $2x10^7$; OD_{600} =0.1, $2x10^8$; OD_{600} =1.0, $2x10^9$cfu/ml). *, P<0.05; **, P<0.01; ***, P<0.001. n=6.

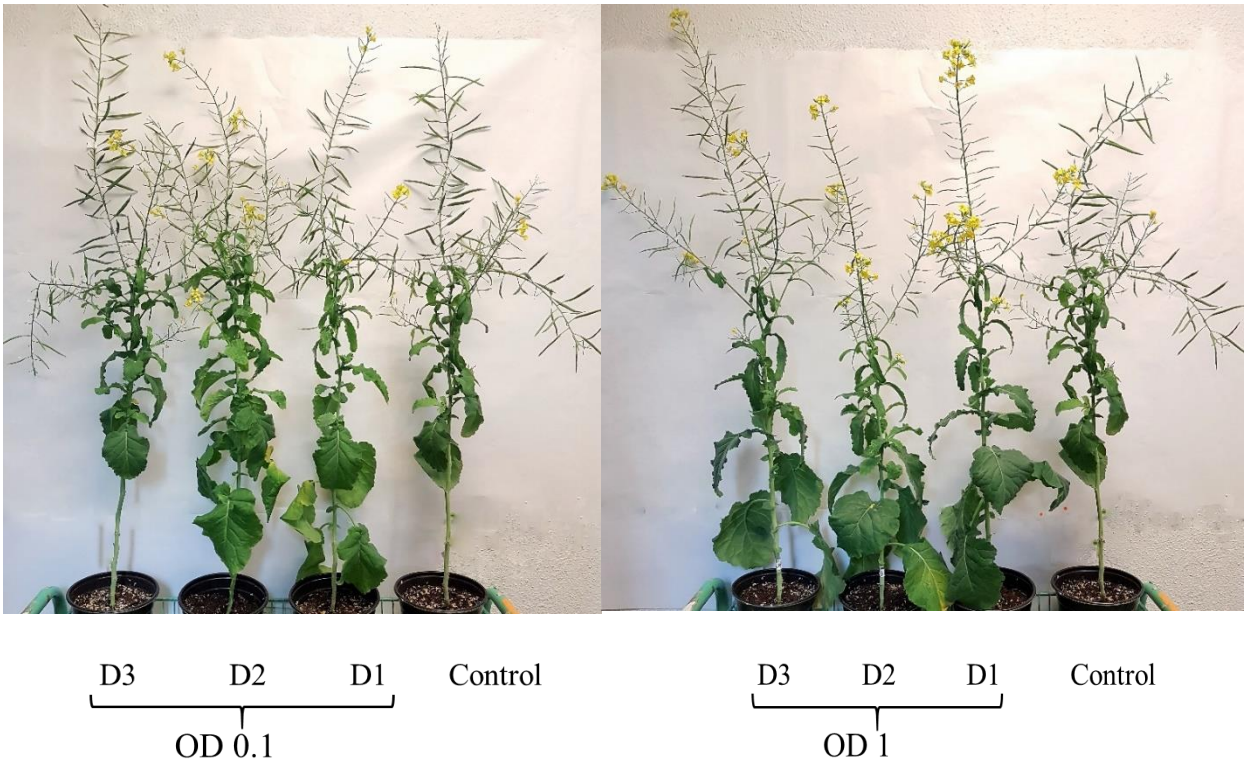


Figure 3.1.3 The impact of multiple applications of PA23 on canola plant development. Plants were treated with one, two or three applications of PA23 at two different concentrations. One representative plant from each treatment group is shown. n=6.

data shown in figure 3.1.4 shows the average values for biomass measurements. Plants treated with PA23 at both concentrations did not show an increase in fresh or dry weight compared to control plants regardless of the number of applications. In addition, no significant increase in shoot length of treated plants compared to control was observed. Plants that were drenched with PA23 culture OD_{600} =1.0 ($2x10^9$cfu/ml) a total of three times had a significantly longer average root length compared to control plants. All other treatment groups showed no increase in root length. Average seed weight was analyzed by extracting seeds from mature pods and determining the total seed weight per plant. This parameter deviated greatly between plants in each treatment group and no significance was observed. Many plants had under-developed pods with no seeds while others had many seeds to collect. At the end of the second trial, several plants showed signs of mildew, which likely contributed to some of the variation observed.

In the third experiment, canola plants were treated with PA23 and the *gacS* mutant at the highest concentration ($2x10^9$cfu/ml) and the effect of one, two and three applications was explored once again. At 13 weeks, pictures of the mature plants were taken prior to analysis (figure 3.1.5). Visually, no obvious differences in size were observed between the treatment groups. After analyzing the fresh and dry weight, none of the treatments resulted in increased biomass compared to the control plants (figure 3.1.6). No significant difference in shoot and root length as well as seed weight was observed (figure 3.1.6). Of the three plant trials examining the PGP effects of PA23 on canola grown to maturity, only trial one showed any appreciable differences between the treated and control plants. Therefore, the first experiment was repeated one more time to validate the original findings. Figure 3.1.7 shows one representative of each of the treated and control plants before biomass analysis. Visually, there were no discernable size differences between the treatment groups and control plants. Fresh weight was

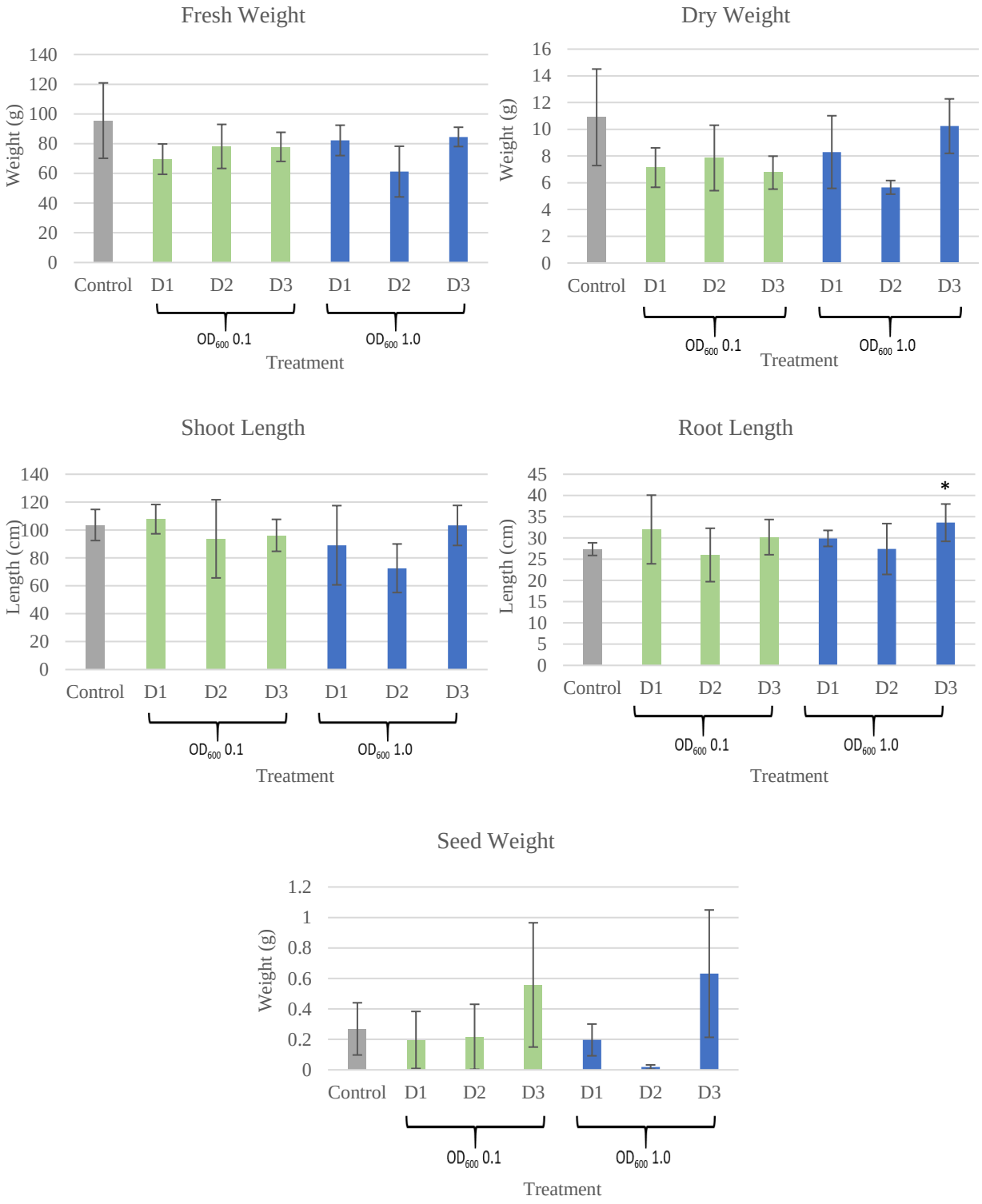


Figure 3.1.4 The effect of the PA23 wt on canola biomass, shoot and root development and seed weight. Plants were treated with one, two or three applications of bacteria at two different concentrations and analyzed after 13 weeks of growth. (OD_{600}=0.1, $2x10^8$cfu/ml and OD_{600}=1.0, $2x10^9$cfu/ml). *, $P<0.05$. n=6.

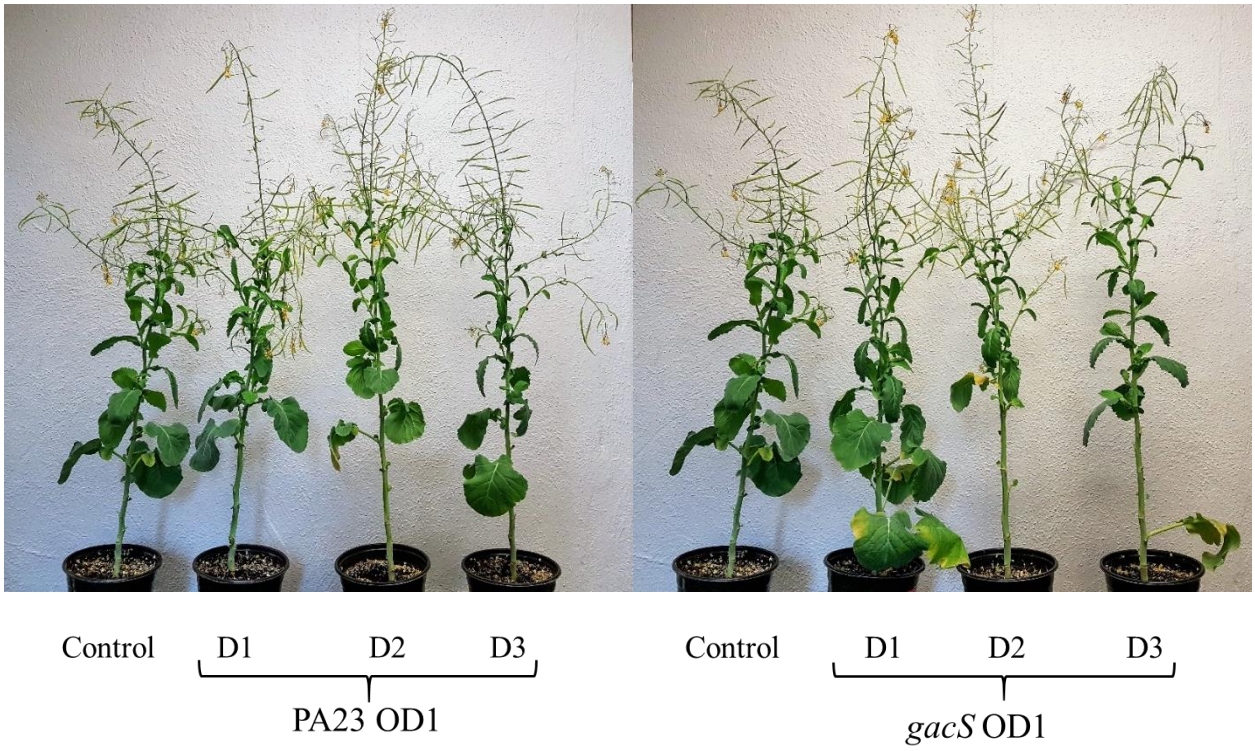


Figure 3.1.5 The impact of multiple applications of PA23 and the *gacS* mutant on canola plant development. Plants were treated with one, two or three applications of bacteria at a concentration of OD_{600}=1.0. One representative plant from each treatment group is shown. n=6.

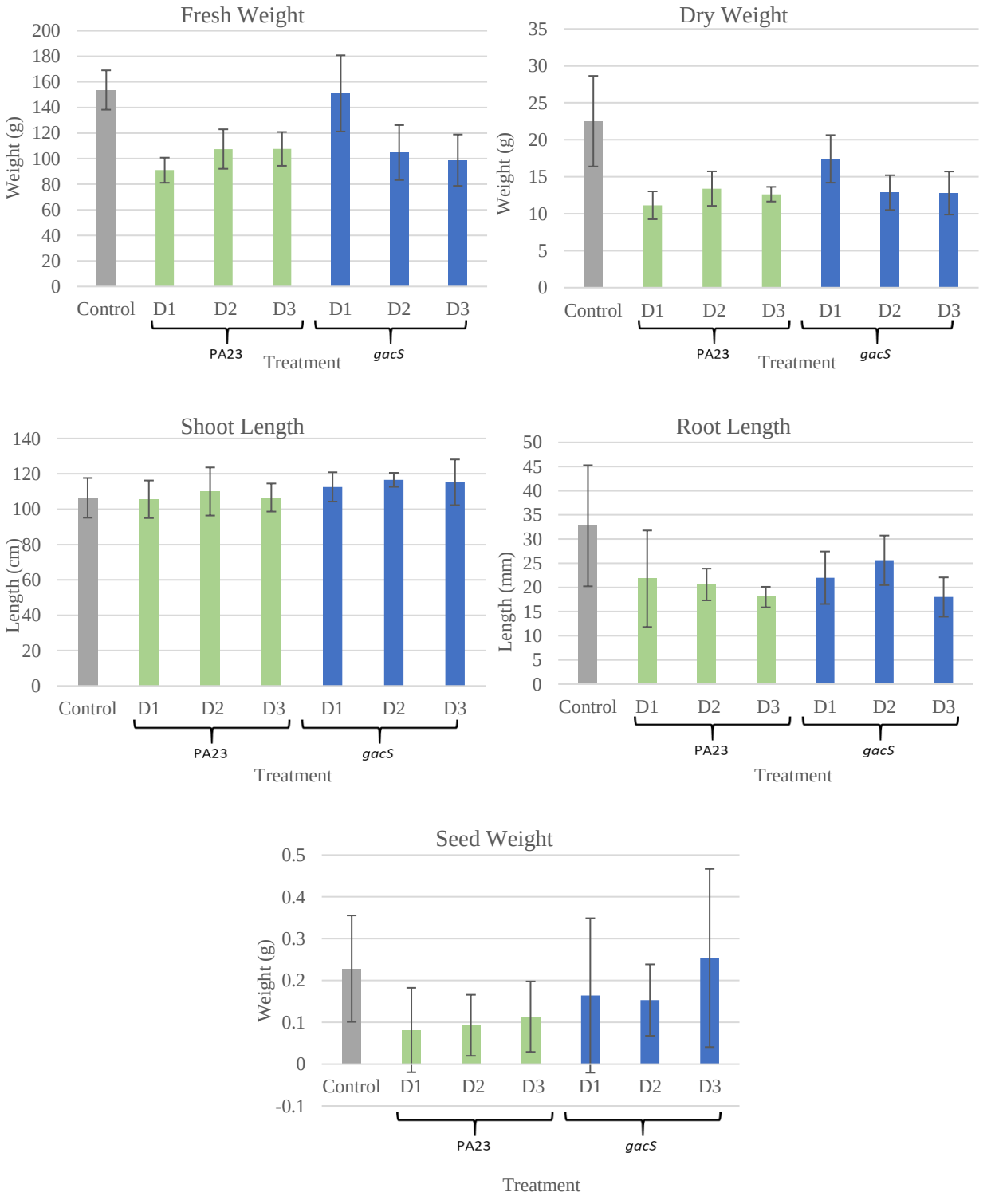


Figure 3.1.6 The effect of PA23 wt and the *gacS* mutant on canola biomass, shoot and root development, and seed weight. Plants were treated with one, two and three applications of bacteria at a concentration of OD_{600}=1.0 and analyzed after 13 weeks of growth. n=6.

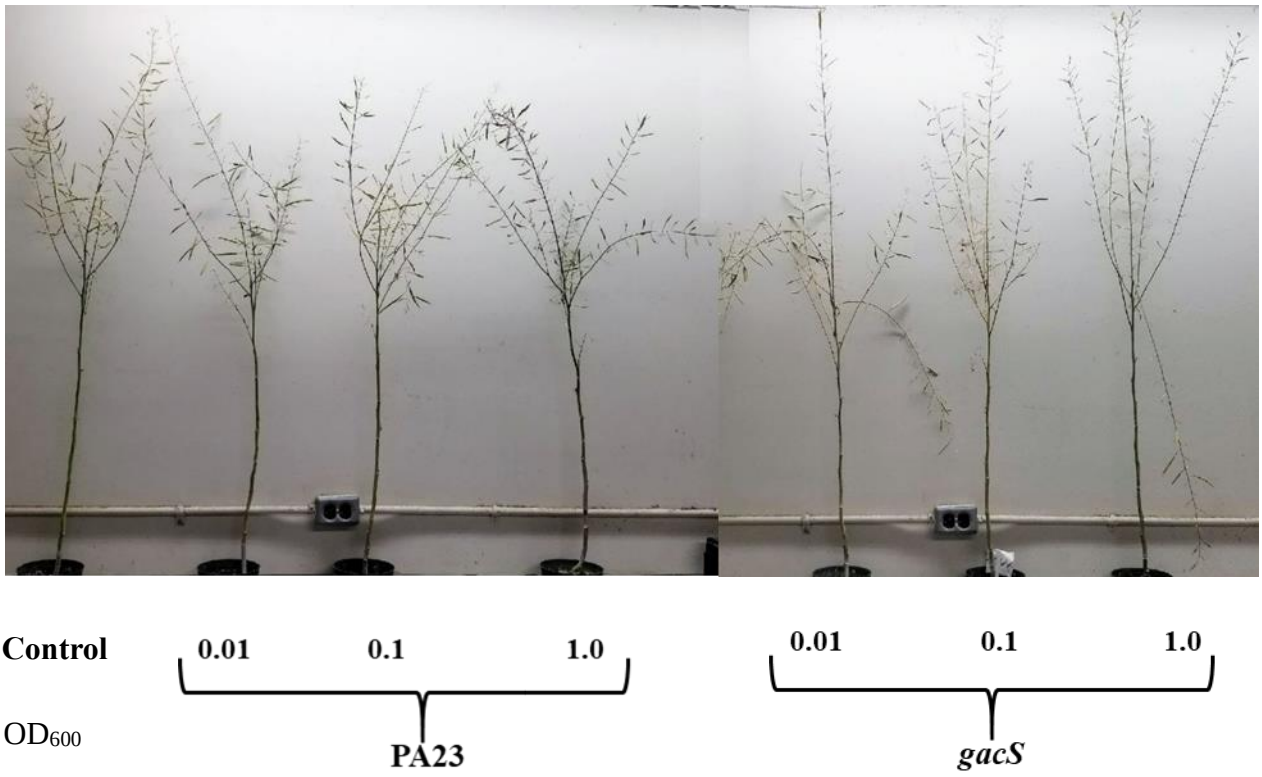


Figure 3.1.7 Picture of 13-week old canola plants before harvesting for biomass analysis. Three different concentrations of PA23 and the *gacS* mutant (OD_{600} =0.01, $2x10^7$; OD_{600} =0.1, $2x10^8$; OD_{600} =1.0, $2x10^9$cfu/ml) were used for plant drenching. n=6.

significantly increased in plants treated with PA23 OD_{600}=0.01 and OD_{600}=1.0; but only the latter treatment resulted in significantly higher dry weight (figure 3.1.8). Increased shoot length was observed in all PA23 treated plants, and the higher concentration (OD_{600}=1.0) resulted in significantly increased root length. None of the *gacS* mutant treatments brought about differences in plant biomass. Furthermore, seed weight varied greatly between all the treatment groups and control plants with no significant difference.

3.2 Canola seedlings show limited PGP 7, 14 and 21 days after irrigation with PA23 but not the *gacS* mutant.

To examine if irrigation with the PA23 wt and the *gacS* mutant promotes growth early in canola development, plants were treated one week after sowing and analyzed at 7, 14 and 21 dpt. Three different concentrations were tested, $2x10^7$cfu/ml (OD_{600}0.01), $2x10^8$ cfu/ml (OD_{600}0.1) and $2x10^9$ cfu/ml (OD_{600}1.0), to observe if PGP was concentration dependent. At 7 dpt, there were no obvious differences between treatment groups upon visual inspection (figure 3.2.1A). Quantitative analysis of plant biomass revealed no significant difference in fresh and dry weight of treated plants compared to control plants at 7 dpt (figure 3.2.1B). Similar findings were obtained for root and shoot length (figure 3.2.1B). Visual comparison of 14 dpt canola plants treated with PA23 and the *gacS* mutant showed no major differences in size compared to control plants (figure 3.2.2). Canola seedlings harvested at 14 dpt exhibited no significant increase in fresh and dry weight for the PA23- and the *gacS* mutant-treated plants compared to control plants (figure 3.2.3). Similarly, there was no significant increase in root and shoot length (figure 3.2.3). Plants harvested 21 dpt showed similar variation in biomass. Visually, there was no major size increase for plants treated with bacteria compared to controls

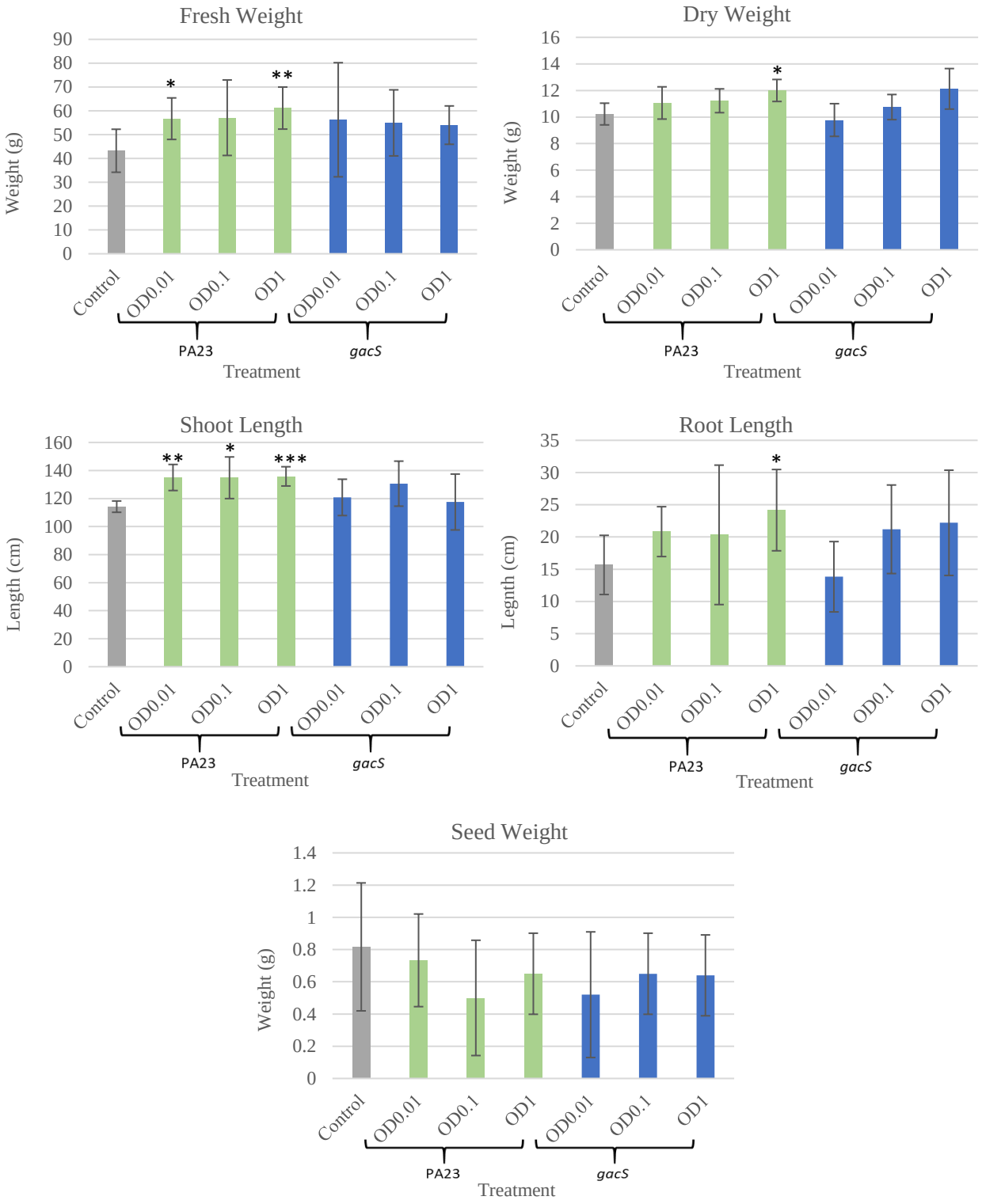


Figure 3.1.8 The effect of the PA23 wt and the *gacS* mutant on canola biomass, shoot and root development, and seed weight. Plants were treated with three different concentrations of bacteria and analyzed after 13 weeks of growth. *, P<0.05; **, P<0.01; ***, P<0.001. n=6.

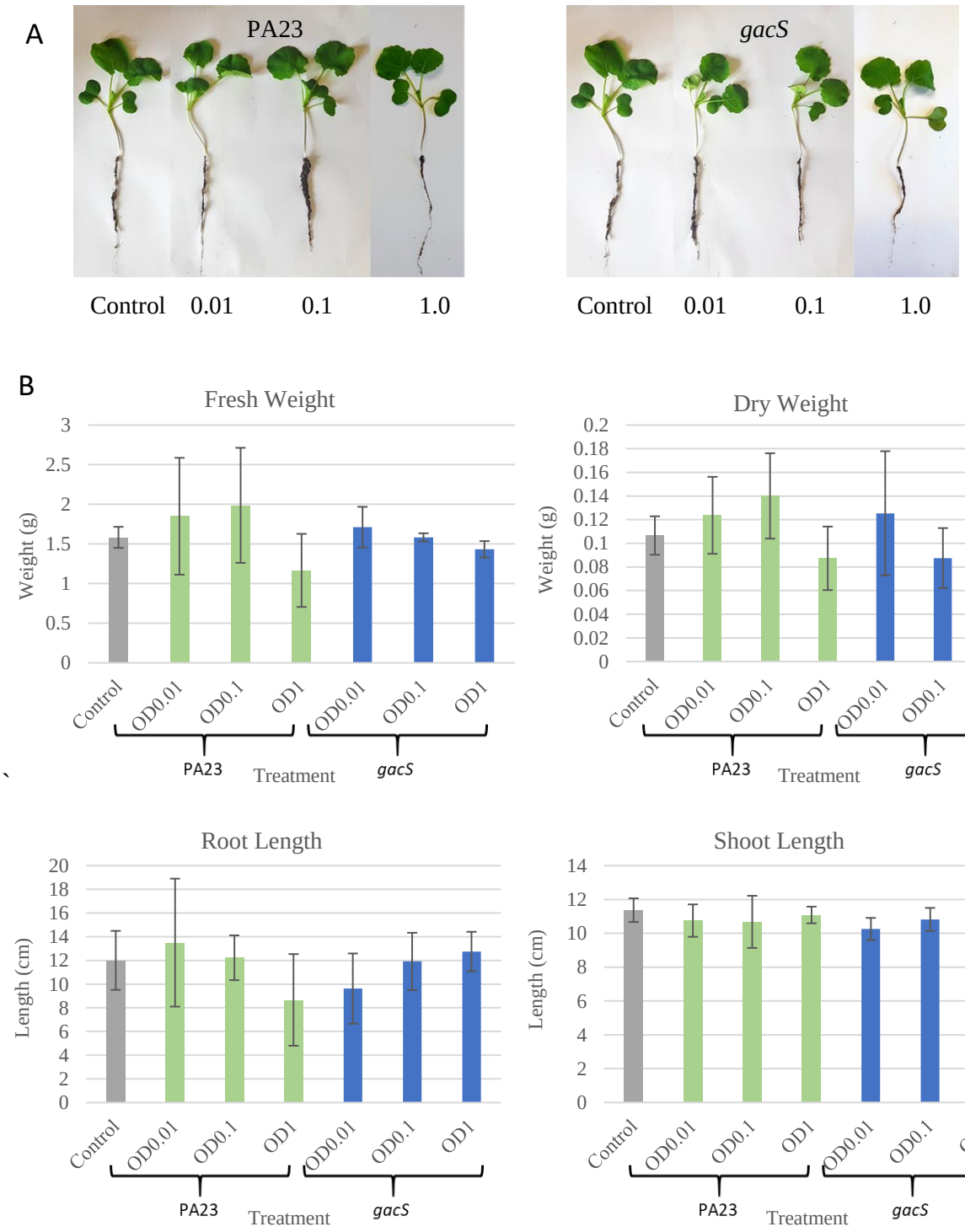


Figure 3.2.1 Biomass of canola seedlings harvested at 7 dpt. A. Picture of a representative canola plant from the three treatment groups: OD_{600} =0.01 ($2x10^7$ cfu/ml), OD_{600} =0.1 ($2x10^8$ cfu/ml), OD_{600} =1.0 ($2x10^9$ cfu/ml) B. Average fresh and dry weight and root and shoot length measurements of the canola seedlings. n=8.

PA23

Control 0.01 0.1 1.0

gacS

Control 0.01 0.1 1.0

Figure 3.2.2 Picture of a representative canola plant (14 dpt) from each treatment: OD_{600}=0.01 ($2x10^7$cfu/ml), OD_{600}=0.1 ($2x10^8$cfu/ml), OD_{600}=1.0 ($2x10^9$cfu/ml). n=6.

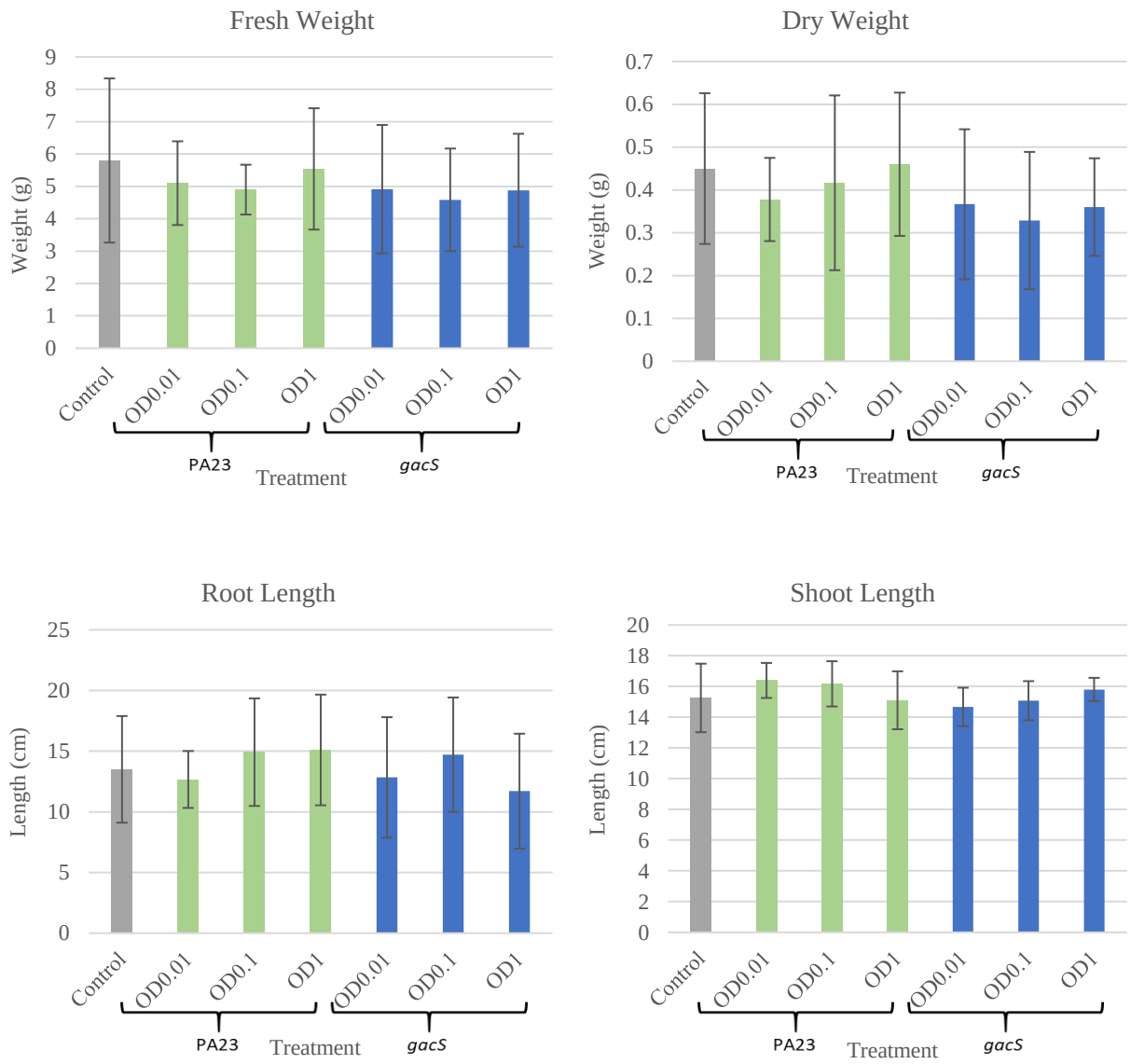


Figure 3.2.3 The effect of the PA23 wt and the *gacS* mutant on canola seedling biomass and shoot and root development. Seedlings were treated with three different concentrations of bacteria and analyzed 14 dpt; OD_{600}=0.01 ($2x10^7$cfu/ml), OD_{600}=0.1 ($2x10^8$cfu/ml), OD_{600}=1.0 ($2x10^9$cfu/ml). n=6.

(figure 3.2.4). These findings extended to the quantitative analysis of fresh and dry weight and root length (figure 3.2.5). The only significant difference observed was shoot length for the PA23 (OD_{600}=1.0) treated plants, but this increase was marginal.

3.3 Lettuce plants show variable PGP when drenched with PA23 and the *gacS* mutant at different concentrations.

Initial studies describing PA23-mediated PGP were performed using lettuce plants. Therefore, in the next set of experiments, PA23 and the *gacS* mutant were tested on lettuce. Since the lettuce seedlings were too fragile to transfer after one week, they were grown for three weeks and then transferred to large pots at which point bacterial treatments were applied. Lettuce plants were harvested after 5 weeks of growth, which is the recommended time of maturity stated on the seed package (Heritage, Brandon, MB, CA). Figure 3.3.1 depicts one plant from each treatment group prior to harvesting. Visual observation of lettuce plants revealed no major differences in plant size. In subsequent biomass analysis, fresh and dry weights were variable (figure 3.3.2). The experiment was repeated three times and all had similar outcomes. Plants treated with PA23 at an OD_{600}= 0.01 showed a significantly increased average shoot length compared to controls plants; however, this was not observed for all three experiments. Plants treated with the *gacS* mutant (OD_{600}=0.1) also showed an increase in average shoot length compared to control plants; however, this increase was only observed in two of the three trials. Root length consistently varied across the treatment groups and none of the plants displayed a longer average root length compared to control plants.

PA23

Control 0.01 0.1 1.0

gacS

Control 0.01 0.1 1.0

Figure 3.2.4 Picture of a representative canola plant from each treatment 21 dpt; OD_{600}=0.01 ($2x10^7$cfu/ml), OD_{600}=0.1 ($2x10^8$cfu/ml), OD_{600}=1.0 ($2x10^9$cfu/ml). n=6.

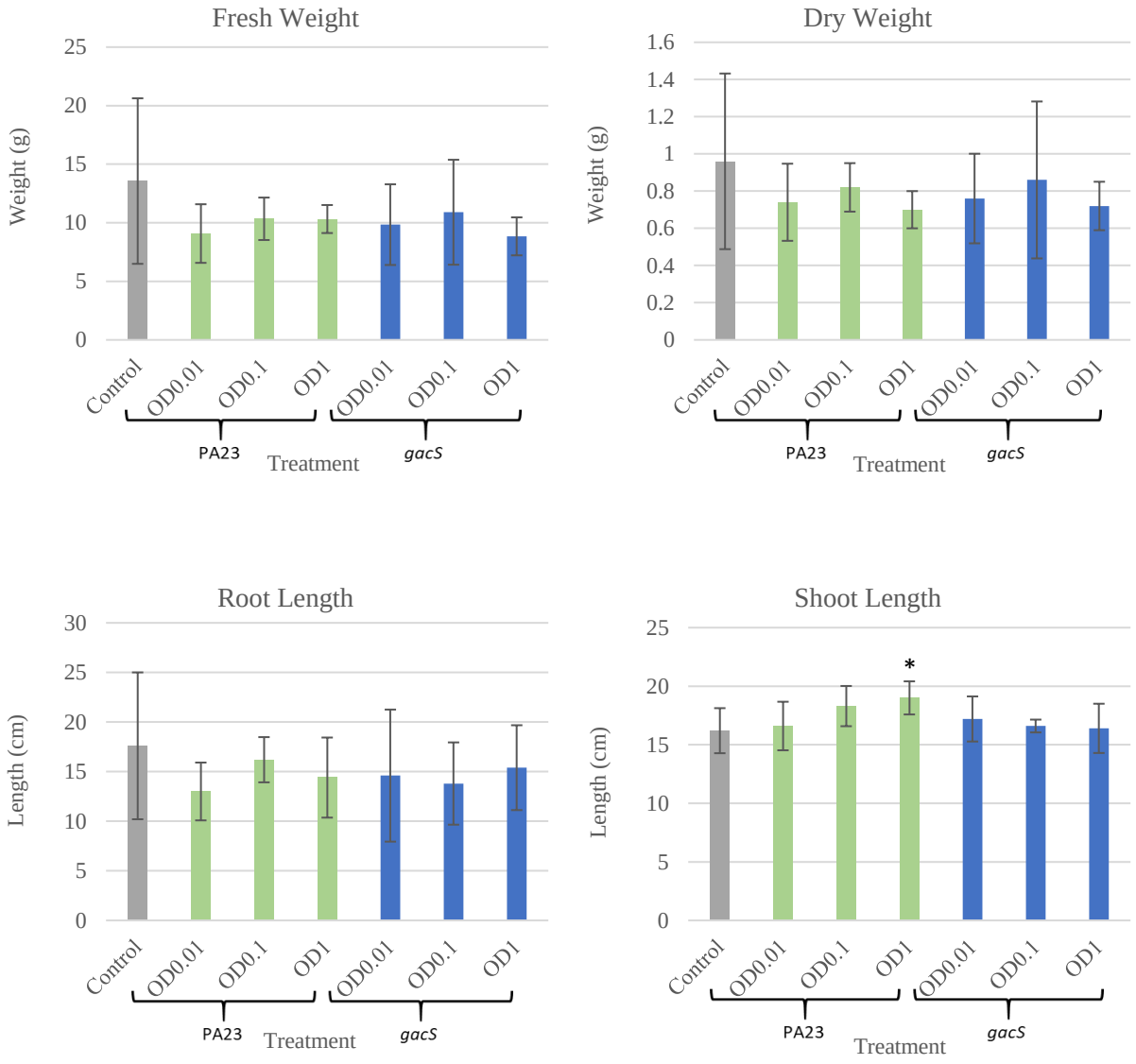


Figure 3.2.5 The effect of the PA23 wt and the *gacS* mutant on canola seedling biomass and shoot and root development. Seedlings were treated with three different concentrations of bacteria and analyzed 21 dpt; OD_{600}=0.01 ($2x10^7$cfu/ml), OD_{600}=0.1 ($2x10^8$cfu/ml), OD_{600}=1.0 ($2x10^9$cfu/ml). (*, P<0.05). n=6.

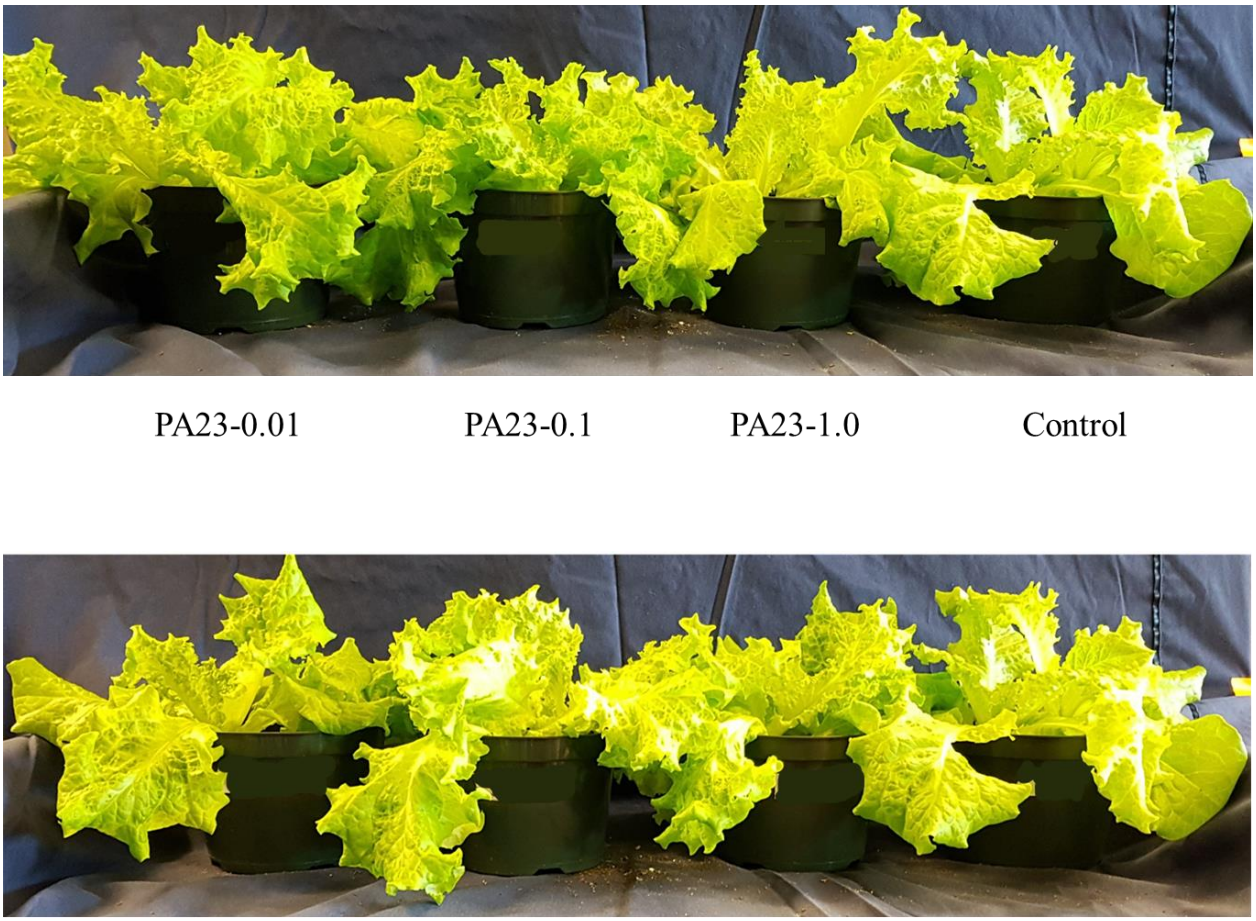


Figure 3.3.1 Pictures of treated lettuce before harvest (5 weeks old). Plants were treated with three different concentrations of either the PA23 wt or the *gacS* mutant; OD_{600}0.01 ($2x10^7$cfu/ml), OD_{600}0.1 ($2x10^8$cfu/ml) and OD_{600}1.0 ($2x10^9$cfu/ml). n=6.

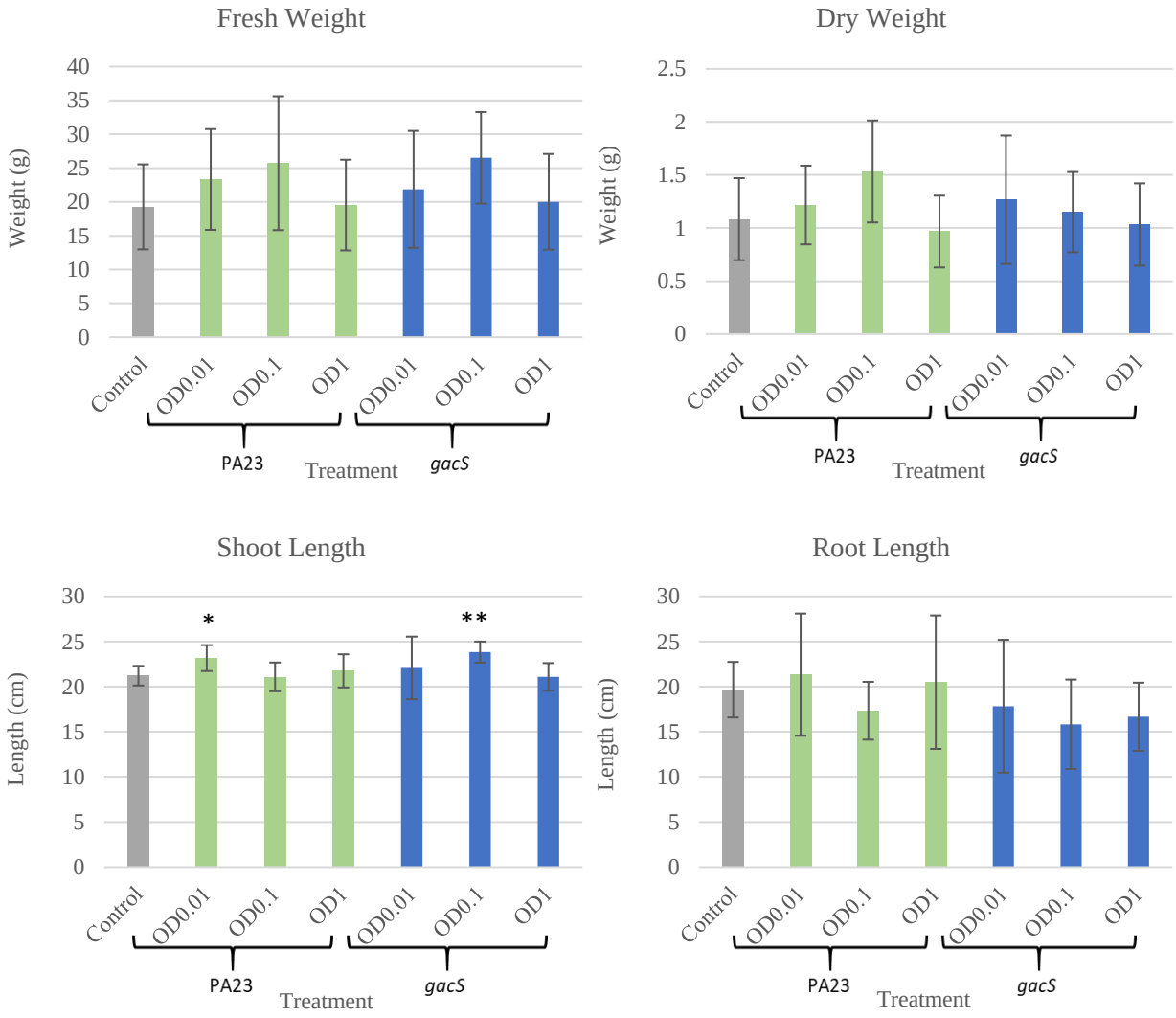


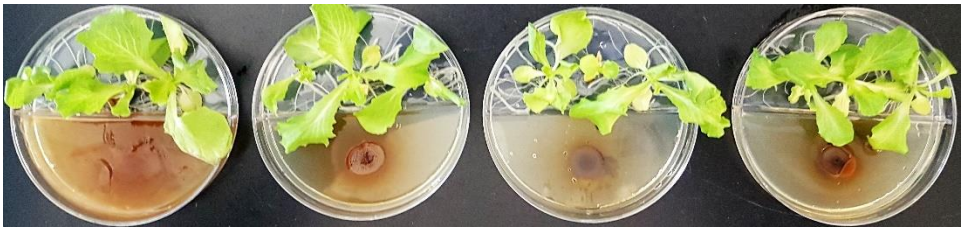
Figure 3.3.2 The effect of the PA23 wt and the *gacS* mutant on lettuce biomass and shoot and root development. Plants were treated with three different concentrations of bacteria and analyzed after 5 weeks of growth; OD_{600}=0.01 ($2x10^7$cfu/ml), OD_{600}=0.1 ($2x10^8$cfu/ml), OD_{600}=1.0 ($2x10^9$cfu/ml). (*, P<0.05; **, P<0.01). n=6.

3.4 Lettuce exposed to volatiles produced by the PA23 wt and the *gacS* and *hcn* mutants showed increased biomass.

To observe the effect that the volatiles produced by PA23 and the *gacS* and *hcn* mutants have on lettuce plants, we compared plant biomass of lettuce seedlings exposed to the three bacterial strains in an enclosed system. Since the PA23 wt produces the toxic volatile HCN, the HCN-deficient *gacS* and *hcn* mutants were incorporated to observe if HCN has a negative effect on plant growth. Figure 3.4.1 shows the lettuce plants growing on agar plates before biomass analysis. Visually, the root and shoots of lettuce plants exposed to PA23 and the *gacS* and *hcn* mutants appear larger than the control plants (Figure 3.4.2A). After measuring several parameters of plant biomass, lettuce seedlings exposed to PA23 and the *gacS* and *hcn* mutants showed a significant increase in fresh and dry weight (Figure 3.4.2B). Lettuce seedlings exposed to the *gacS* mutant has significantly increased root length (Figure 3.4.2B). Finally, lettuce seedlings exposed to PA23 and the *hcn* mutant produced significantly longer shoots compared to the control. Experiments were repeated twice and the same findings were observed both times.

Control

PA23



hcn

gacS

Figure 3.4.1 Three-week old lettuce plants before harvesting for biomass analysis. Plants were grown for 14 days in enclosed vessels in the presence of PA23 and the HCN-deficient *gacS* and *hcn* mutants. Note: plants exposed to bacterial volatiles produced by all three strains appear larger than the control plants. n=12-15.

Figure 3.4.2. Three-week old lettuce plants after harvesting for biomass analysis. Plants were grown for 14 days in enclosed vessels in the presence of PA23 and the HCN-deficient *gacS* and *hcn* mutants. Note: plants exposed to bacterial volatiles produced by all three strains show longer roots and shoots compared to control plants. n=12-15.

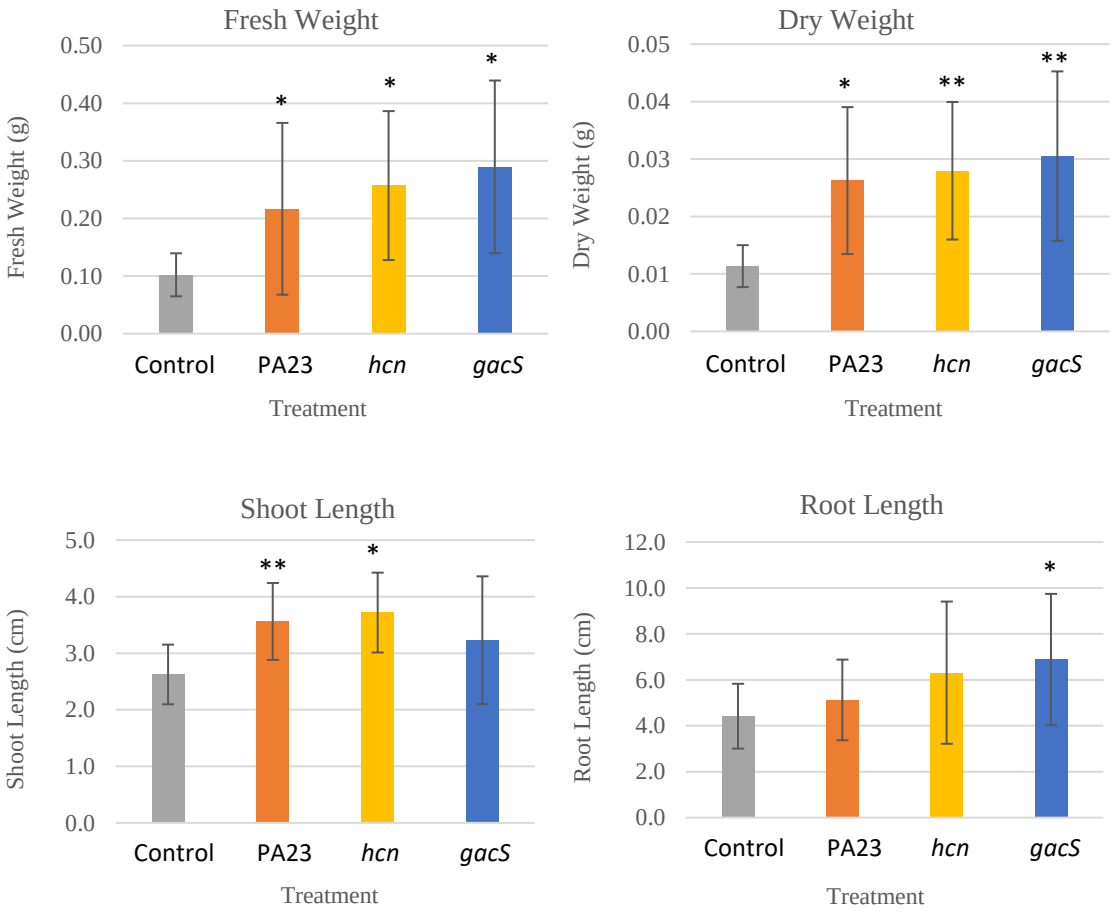


Figure 3.4.3. Differences in biomass of three-week-old lettuce seedlings exposed to volatiles produced by PA23 and the *gacS* and *hcn* mutants. One-week old lettuce seedlings were exposed to PA23, the *gacS* or *hcn* mutants (OD_{600}=0.1) inoculated on the opposite side of a split plate and grown for two weeks in an enclosed system. **A.** Lettuce seedlings exposed to PA23, the *gacS* and *hcn* mutants appeared to be larger than control plants. **B.** Averages from quantitative analysis of plant fresh and dry weight and root and shoot length. (*, P<0.001; **, P<0.0001). n=12-15.

3.5 Dry formulated PA23 cells maintain higher viability during storage at 4ºC compared to liquid formulations.

In order to commercialize PA23 as a biocontrol product, the cells must be prepared in a formulation that maintains cell viability for extended periods of time. It is suggested that a good formulation can remain viable for 1-2 years (Bashan et al. 2014). Therefore, this is a challenge that must be overcome. In addition, the formulation must be conducive to the mode of application. Since PA23 antagonism against *S. sclerotiorum* takes place in the phyllosphere, a vaporized spray is a practical application method. Various liquid and dry formulations were selected that would support a spray application. Infection assays conducted *in planta* revealed that PA23 at a concentration of $2x10^8$cfu/ml suppresses *S. sclerotiorum* (Savchuk and Fernando, 2004); therefore, a storage concentration above this target was desired. Figure 3.5.1 depicts the cfu/ml determined for all liquid PA23 cell formulations stored for 48 weeks at 4ºC (table 2.5.1). During one year of storage, the number of viable cells in the liquid formulations decreased drastically from a starting concentration of $1x10^{10}$cfu/ml to $1x10^7$cfu/ml. As such, none of the formulations proved to be superior in terms of maintaining cell viability.

Figure 3.5.2 shows the viability of the dried PA23 cell suspensions analyzed during 48 (batch 1) and 24 (batch 2) weeks of storage at 4ºC (table 2.5.2). In the first batch, PA23 cells cultured in either LB or $M9^+$ media and formulated with lactose had an initial concentration of $1x10^{10}$cfu/ml prior to lyophilization. Over 48 weeks of storage, the viability of the LB cultured cells decreased from $8.9x10^8$cfu/ml (immediately post lyophilization) to $8.5x10^7$cfu/ml (week 48). After freeze drying, PA23 cells cultured in $M9^+$ media (with lactose) showed a cell concentration of $5x10^7$cfu/ml; this viability was maintained throughout 48 weeks of storage.

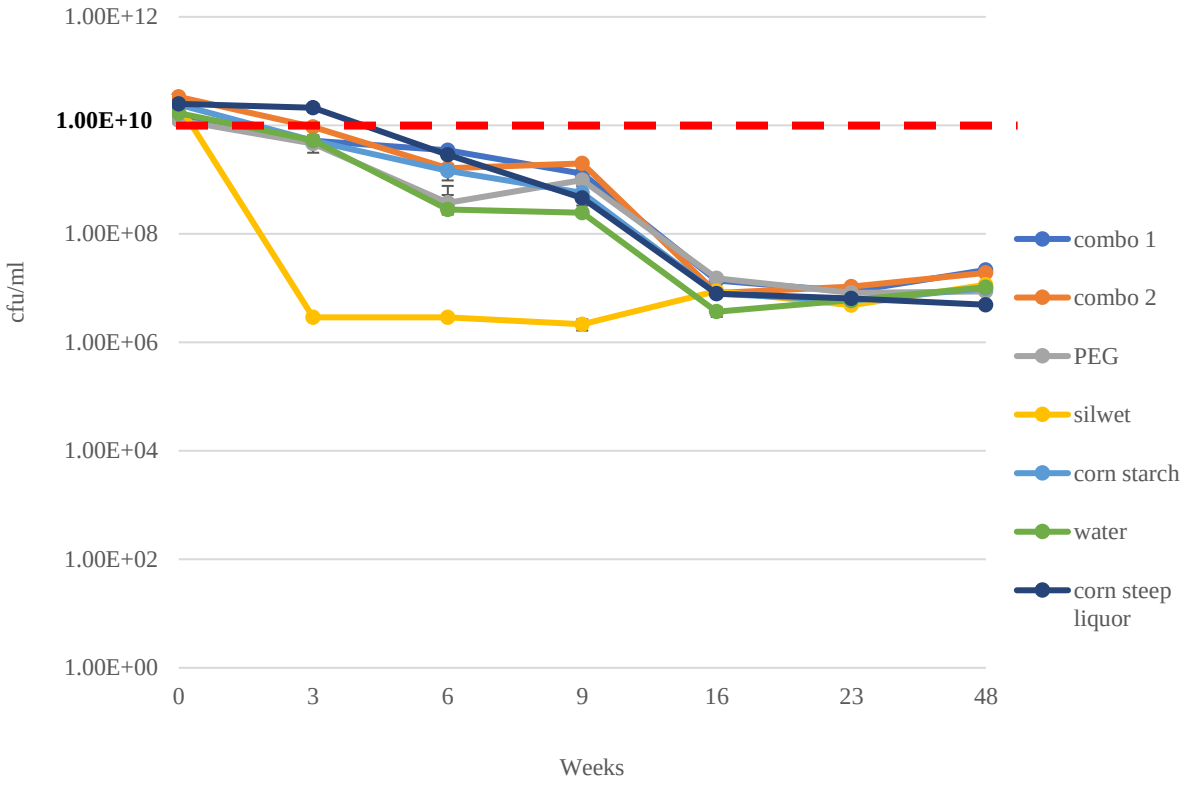


Figure 3.5.1 Viability determination of the liquid formulations containing PA23 cells suspended in LB broth with various additives. Cell formulations were stored at 4ºC and cfu/ml was analyzed at various time points for 48 weeks. The red line defines the target sample concentration prior to storage.

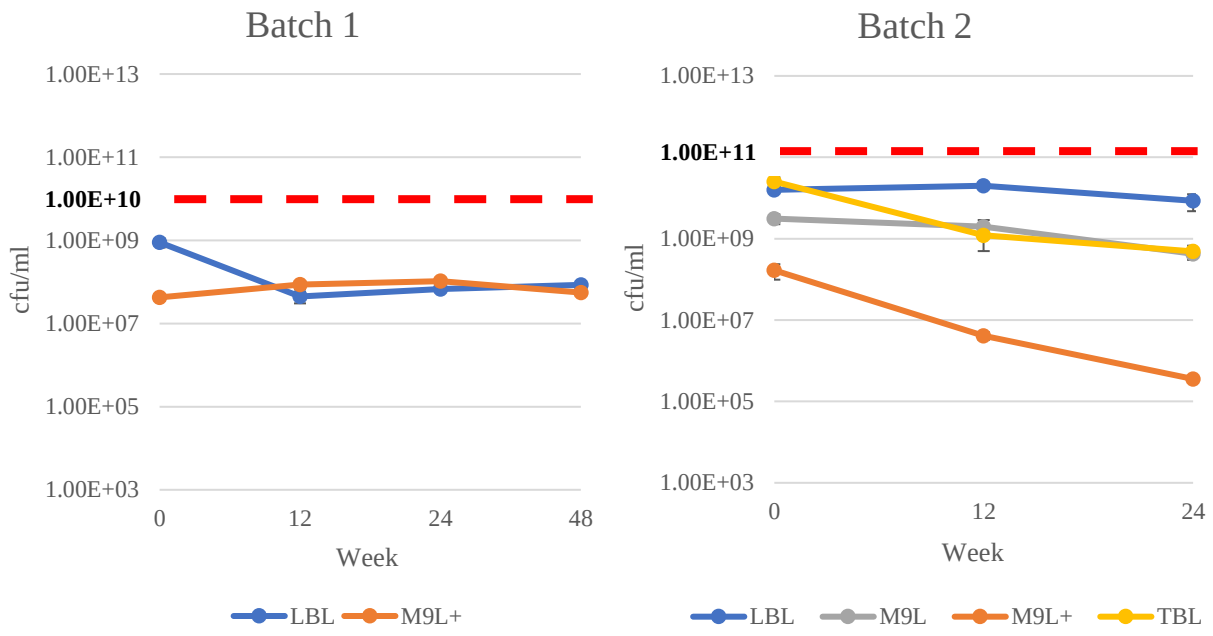


Figure 3.5.2 Viability determination of the dry formulations containing PA23 cells cultured in different types of media plus lactose (150g/L). Two independent experiments were conducted labelled Batch 1 and Batch 2. Viability analysis was undertaken over the course of 48 weeks (Batch #1) and 24 weeks (Batch #2). The red line defines the sample target concentration prior to lyophilization.

Analysis of PA23 cells cultured in either medium (LB or $M9^+$) and lyophilized with lactose showed similar viability after 48 weeks of storage at 4^oC (Figure 3.5.2; Batch 1).

It has been reported that cultures containing a higher density of cells collected during the stationary phase exhibit improved survival during drying and storage (Bashan et al. 2014). To increase the viability of the second batch of samples, PA23 cells were collected at the beginning of the stationary phase and cultures were concentrated to a higher cell density ($1x10^{11}$cfu/ml) prior to freeze drying. In addition, M9 and TB media were included. In figure 3.5.2, the right panel shows the second batch of freeze-dried samples. PA23 cells were cultured in LB (8h), M9 (10h), $M9^+$ (16h) and TB (7h). After 24 weeks of storage, PA23 cells cultured in LB and freeze dried with lactose maintained the highest viability ($8.5x10^9$cfu/ml) upon storage at 4^oC, with an initial concentration of $1.6x10^{10}$cfu/ml immediately after lyophilization. PA23 cells cultured in TB displayed the highest viability after lyophilization ($2.5x10^{10}$cfu/ml); however, cell viability decreased substantially after 24 weeks to a concentration of $4.9x10^8$cfu/ml. Similarly, M9 cultured cells dropped from a concentration of $3.1x10^9$cfu/ml (immediately after lyophilization) to $4.3x10^8$cfu/ml (week 24). The biggest loss in viability was seen with $M9^+$ cultured PA23 cells, which decreased from $1.7x10^8$cfu/ml immediately after lyophilization to a concentration of $3.6x10^5$cfu/ml after 24 weeks of storage. Another set of $M9^+$ cells cultured with lactose was generated such that a higher starting concentration was obtained after lyophilization; however, the cells did not survive. Since, the $M9^+$ cultured cells showed such a low viability after 24 weeks, they were not tested in the following phenotypic and infection assays.

3.6 Secondary metabolite activity levels of liquid and dry PA23 formulations are similar to freshly cultured PA23.

To observe secondary metabolite production, phenotypic assays were performed to compare the PA23 cell formulations stored over time to freshly cultured cells. Figure 3.6.1 displays the protease (panel I), autoinducer (panel II) and antifungal activity (panels III and IV) of each liquid formulation (48 weeks storage) and freshly cultured PA23. Visually, the proteolytic zones surrounding the liquid formulations appeared slightly larger compared to fresh PA23 (figure 3.6.1, panel I). However, there were no discernable differences observed for autoinducer and antifungal activity. These trends were supported by statistical analysis of the measured zones of activity surrounding each sample shown in table 3.6.1.

Figure 3.6.2 shows the phenotypic assays for batch 1 and 2 of the dry formulations stored for 48 and 24 weeks, respectively. There were no differences in protease (panel I), autoinducer (panel II) and fungal activity (panels III and IV) for the dry formulations compared to freshly cultured PA23 (table 3.6.2). For the third set of dry formulated PA23 samples, cells were cultured in TB and freeze dried with lactose. For all three phenotypic assays, no significant differences were observed between the stored dry sample and fresh PA23 (figure 3.6.3; table 3.6.3).

3.7 Cotyledon infection assay with *L. maculans* show a larger average lesion size on leaves inoculated with the dry formulations compared to the liquid formulations.

To analyze the biocontrol capabilities of the PA23 cell formulations stored over time, a canola leaf infection assay with *L. maculans* was performed. The formulated cell samples were re-suspended in fresh LB broth and immediately applied onto the wounds generated on the

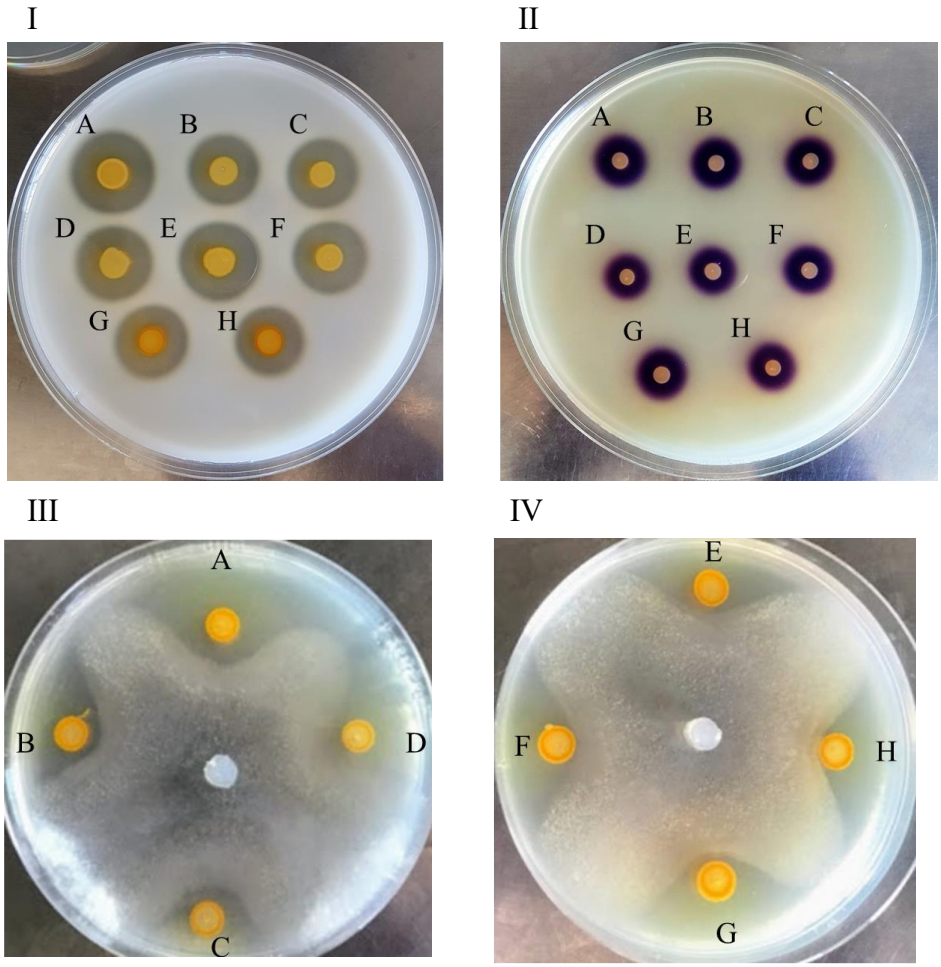


Figure 3.6.1 Phenotypic assays performed for the liquid formulations after 48 weeks of storage at 4^{o}C. Protease (panel I), autoinducer (panel II) and antifungal activity (panels III, IV) were observed. **A.** Combo #1. **B.** Combo #2. **C.** PEG **D.** Silwet. **E.** Corn starch. **F.** H_2O. **G.** CSL. **H.** Fresh PA23. A volume of 5µl ($2x10^9$cfu/ml suspension) was inoculated onto plates and incubated at 28^{o}C.

Table 3.6.1 Phenotypic characteristics of PA23 cells cultured from standard freezer stocks verses liquid formulations after 48 weeks of storage at 4°C.

Sample	Protease-48h*	Protease-72h*	Auto-inducer*	Antifungal*
Fresh PA23	5.1 (0.3)	7.5 (0.7)	4.7 (0.5)	0.6 (1.3)
Combo1	6.7 (0.5)[a]	9.6 (0.7)[a]	5.2 (0.8)[b]	1.4 (0.8)[b]
Combo2	5.6 (0.5)[c]	8.2 (0.6)[d]	4.9 (0.7)[b]	0.3 (1.0)[b]
PEG	5.8 (0.4)[e]	8.2 (0.6)[d]	5.0 (0.9)[b]	0.6 (0.6)[b]
Silwet	6.2 (0.6)[e]	9.3 (0.5)[a]	4.9 (0.7)[b]	0.1 (1.0)[b]
CS	5.8 (0.6)[e]	9.2 (0.6)[a]	4.8 (0.8)[b]	2.1 (1.9)[b]
Water	5.9 (0.7)[c]	8.4 (0.5)[c]	5.0 (0.9)[b]	0.8 (0.1)[b]
CSL	5.7 (0.8)[d]	8.5 (0.7)[c]	5.2 (0.6)[b]	1.8 (1.9)[b]

*Mean (SD) of the zones of activity (mm).
[b] Not significantly different from fresh PA23.
[d] Significantly different from fresh PA23 ($P<0.05$).
[c] Significantly different from fresh PA23 ($P<0.01$).
[e] Significantly different from fresh PA23 ($P<0.001$).
[a] Significantly different from fresh PA23 ($P<0.0001$).

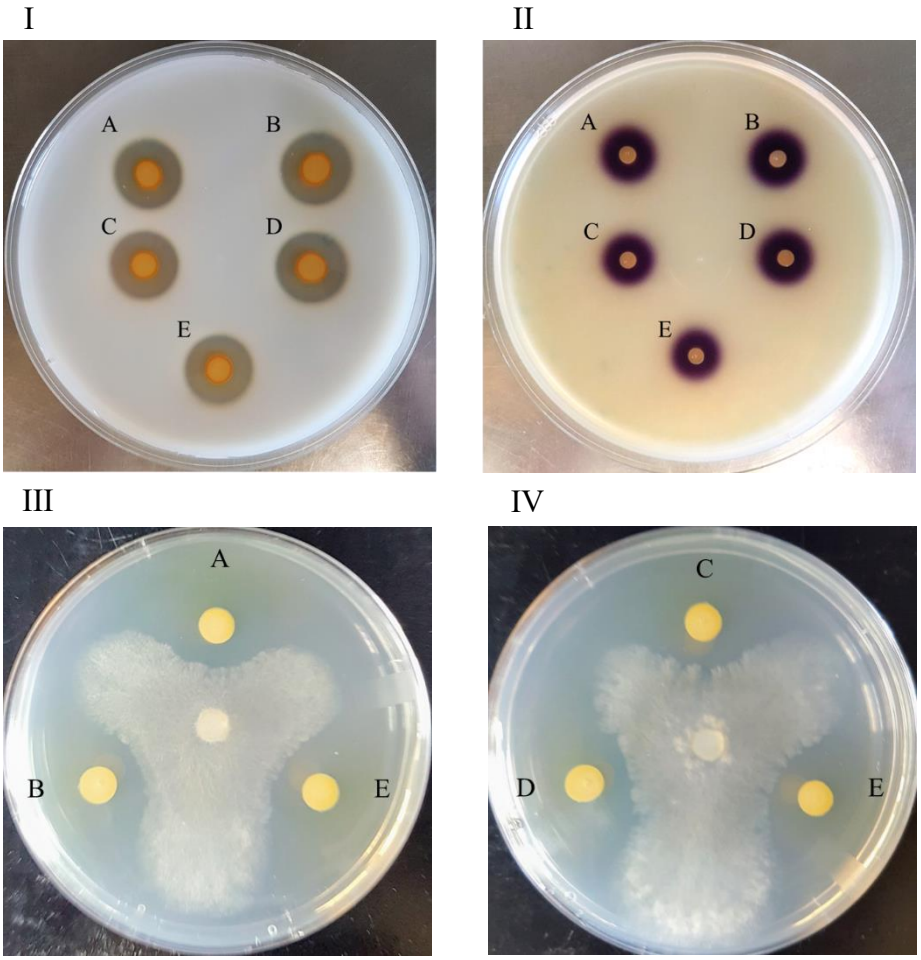


Figure 3.6.2 Phenotypic assays performed for the first and second dry formulation experiments stored for 48 weeks and 24 weeks, respectively at 4^{o}C. Protease (panel I), autoinducer (panel II) and antifungal activity (panels III, IV) were observed. **A.** LBL, 48 weeks storage. **B.** M9^{+}L, 48 weeks of storage. **C.** LBL, 24 weeks of storage. **D.** M9L, 24 weeks of storage. **E.** Fresh PA23. A volume of 5ul (2x10^{9}cfu/ml suspension) was inoculated onto plates and incubated at 28^{o}C.

Table 3.6.2 Phenotypic characteristics of PA23 cells cultured from standard freezer stocks verses dry formulations after 48 (batch 1) and 24 (batch 2) weeks of storage at 4^{o}C.

Sample	Protease-48h*	Protease-72h*	Auto-inducer*	Antifungal*
Fresh PA23	7.3 (0.5)	10.3 (0.5)	8.2 (0.4)	9.2 (0.2)
LBL (48weeks)	7.4 (0.7)	10.8 (0.6)	8.7 (0.7)	8.8 (1.8)
M9^{+}L (48 weeks)	7.4 (0.7)	10.6 (0.7)	8.7 (0.7)	8.7 (1.2)
LBL (24 weeks)	7.5 (0.5)	10.4 (0.5)	8.5 (0.5)	9.0 (1.3)
M9L (24 weeks)	7.2 (0.6)	10.3 (0.5)	8.5 (0.5)	9.2 (0.8)

*Mean (SD) of the zones of activity (mm).
There was no significant difference observed between any of the samples.

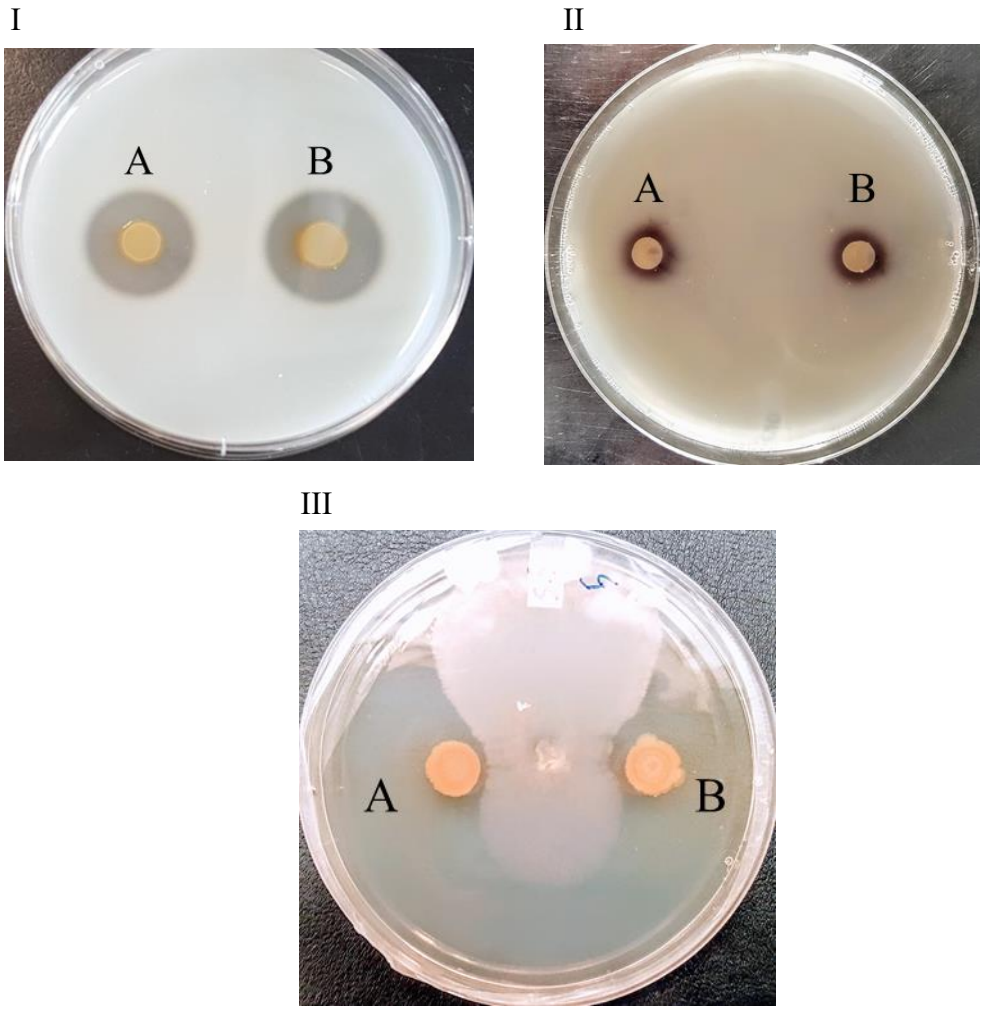


Figure 3.6.3 Phenotypic assays performed for the third set of dry formulations after 24 weeks of storage at 4^{o}C. Protease (panel I), autoinducer (panel II) and antifungal activity (panel III) were observed. **A.** TBL. **B.** Fresh PA23. A volume of 5µl (2x10^{9}cfu/ml suspension) was inoculated onto plates and incubated at 28^{o}C.

Table 3.6.3 Phenotypic characteristics of PA23 cells cultured from standard freezer stocks verses dry formulations after 24 weeks of storage at 4ºC.

Sample	Protease-48h*	Protease-72h*	Auto-inducer*	Antifungal*
Fresh PA23	3.7 (0.4)	6.8 (0.8)	3.4 (0.6)	4.0 (0.7)
TBL (24weeks)	4.1 (0.2)	6.8 (0.8)	3.2 (0.4)	3.8 (0.8)

*Mean (SD) of the zones of activity (mm).
There was no significant difference observed between any of the samples.

cotyledons prior to fungal inoculation. After 14 days of incubation in a growth chamber, the canola seedlings were analyzed for infection. Figure 3.7.1 shows one representative cotyledon from each treatment. Visually, cotyledons treated with the PA23 dry formulations showed increased infection compared to seedlings treated with the liquid formulations. All the dry formulations showed signs of infection (figure 3.7.2). Plants treated with the liquid formulations exhibited no lesion development suggesting that this method of preparing and storing PA23 is effective for maintaining biocontrol properties.

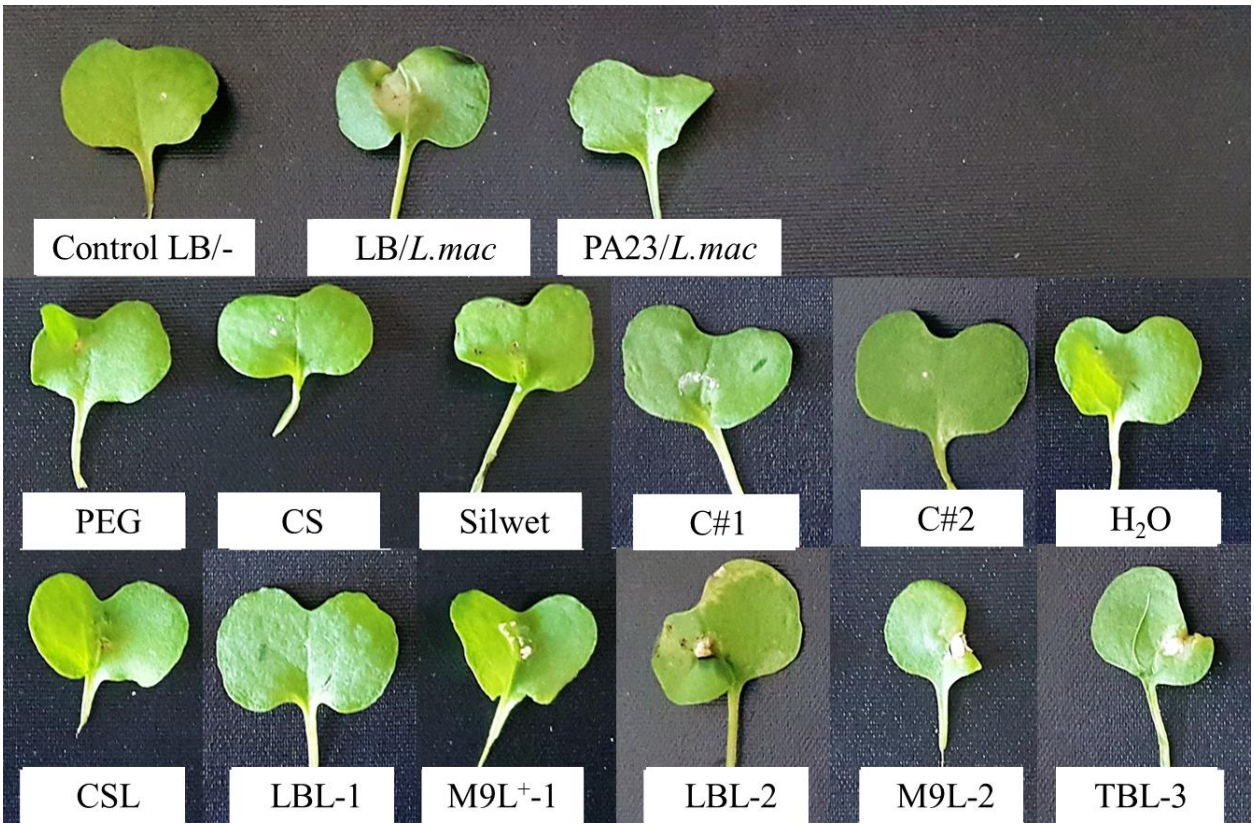


Figure 3.7.1 Cotyledon infection assay with *L. maculans* using the liquid and dry formulation cell suspensions. One representative leaf is shown. After 14 days of incubation, lesion diameters on canola seedlings were measured and analyzed. Liquid formulations, PEG, CS, Silwet¸C#1, C#2, H_2O and CSL and dry formulations, LBL-1 and $M9L^+$-1 (48 weeks) and LBL-2, M9L-2 and TBL-3 (24 weeks) were tested. A volume of 10µl was inoculated into the cotyledon wound and incubated 1h prior to *L. maculans* infection.

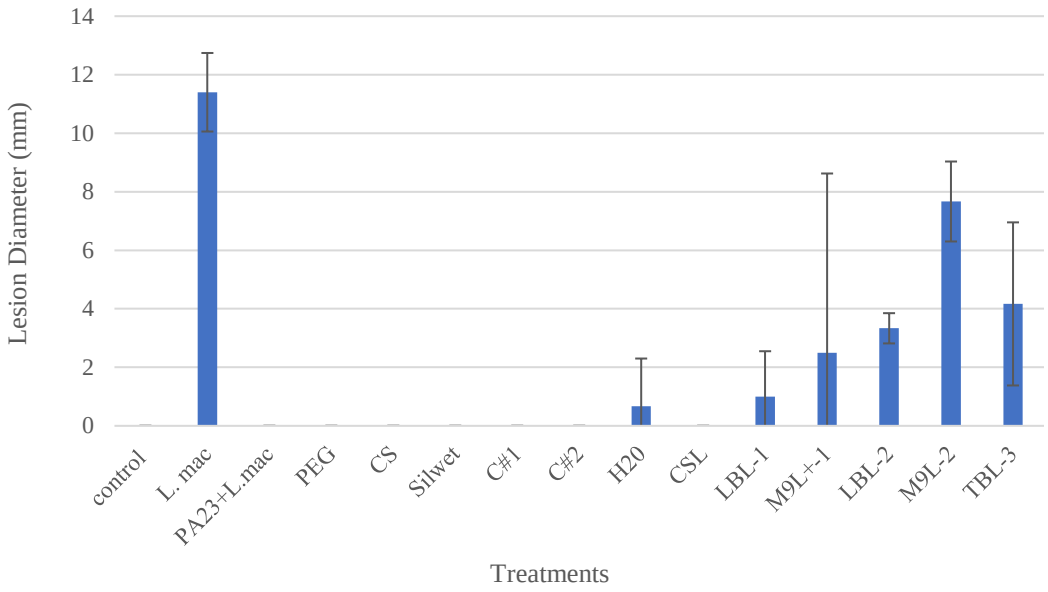


Figure 3.7.2 The biocontrol capability of PA23-based liquid and dry formulations against *L. maculans* infection in canola. Each sample was resuspended in LB broth and inoculated on top of a wound generated in the cotyledon. One hour later, fungal spores were inoculated onto the wound. After 14 days in a growth chamber, lesions on the cotyledons were measured. Liquid formulations: PEG; CS; Silwet; C#1; C#2; H_2O and CSL. Dry formulations: LBL-1 and $M9L^{+}$-1 (48 weeks); and LBL-2, M9L-2 and TBL-3 (24 weeks).

4. DISCUSSION

Canola is an important Canadian crop, contributing on average $26.7 billion dollars per year to the economy and providing over 250,000 jobs (Canola Council of Canada, 2016). Provincially, canola production in Manitoba generates $4.16 billion annually (Canola Council of Canada, 2016). Therefore, protecting the integrity of this crop is a major priority. Biocontrol is an indirect form of PGP and has been studied extensively in PGPR. Fungal pathogens such as *S. sclerotiorum* threaten plant health and possess the ability to cause devastating destruction. PA23 can protect canola against *S. sclerotiorum* infection in both greenhouse and field experiments (Fernando et al. 2007; Savchuk and Fernando 2004; Zhang 2004). Increasing nutrient availability (e.g. nitrogen fixation), synthesizing plant hormones (e.g. IAA) and degradative enzymes (e.g. ACC deaminase) are ways in which PGPR directly encourage plant growth and development. To date, the PGP capabilities of PA23 remain largely unknown. To address this knowledge gap, the effect of PA23 application on canola and lettuce plant biomass was investigated. Lettuce was included along with canola because of its importance and versatility. Ranked in the top ten vegetables consumed in Canada, lettuce is an important crop that can be grown in fields or in controlled greenhouses (Bayer, 2018). Increases in biomass seen in soil experiments with canola and lettuce drenched with PA23 varied between experiments. Obtaining reproducible results with soil-grown plants can be a challenge (Cipriano et al. 2016). Soil environments are subject to limiting conditions that can induce plant stress. For example, high salt content, over watering, lack of nutrients, temperature and pH fluctuations can impact plant health and development. These factors can also influence IAA production in bacteria (Spaepen, Vanderleyden, and Remans 2018). IAA plays a role in virtually all plant development and defense pathways (Ahemad and Kibret 2014). The concentration range of bacterial-mediated IAA that elicits

beneficial effects is believed to be quite narrow; consequently, large fluctuations may negatively impact plant growth (Duca et al. 2014). In addition, high levels of ethylene produced by plants under stress can inhibit IAA signal transduction, lowering the PGP effect (Czarny *et al.*, 2006; Glick *et al.*, 2007; Stearns *et al.*, 2012). Therefore, varying soil environments could affect the production levels of IAA by PA23 and the overall PGP effect.

Findings from an earlier study comparing the biomass of plants inoculated with PA23 cultivated in different growth environments may support this theory (Reimer, 2016). Canola seeds inoculated with PA23 and grown in hydroponic pouches showed increased root length compared to control plants (Reimer, 2016). In contrast, similar growth experiments conducted in soil with PA23 showed no increase in root or shoot length (Reimer, 2016). Therefore, different growth environments may affect the PGP ability of PA23, whether related to IAA production or other PGP properties. Furthermore, it is important to consider that PA23 does not produce ACC deaminase (Reimer, 2016). Under stressed soil conditions (salt or temperature stress) seed inoculation with *P. putida* GR12-2 promoted increased root length and fresh/dry weight compared to control plants (Glick et al. 1997). This increase in biomass was lost when an ACC deaminase-deficient mutant GR12-2/*acd*68 was tested in stressed soil with canola (Glick et al. 1997). ACC deaminase promotes plant growth by lowering ethylene levels in stress-induced environments. With the absence of ACC deaminase in PA23, IAA-induced PGP may be more sensitive to fluctuating growth conditions and stress induced ethylene production.

To further investigate the PGP capabilities of PA23, enclosed plate assays were used to observe the effect of bacterial volatiles on plant growth. Volatile compounds produced by PGPR have been shown to have growth promotion effects on plants. In a 2015 study, tobacco plants exposed to *P. fluorescens* SS101 volatiles showed increased biomass in an enclosed divided plate

assay (Park et al. 2015). PA23 was previously reported to produce volatiles that elicit antifungal activity against *S. sclerotiorum*; therefore, these compounds may also have PGP potential (Fernando et al. 2005). Employing the same enclosed split plate method outlined in Park *et al.* (2015), 7-day old lettuce seedlings were exposed to PA23 volatiles. Exposed plants showed significant increases in plant biomass compared to control plants. Since PA23 produces HCN, which could be toxic to the plants, the *gacS* and *hcn* mutants were tested. After two weeks of growth, there were no significant differences in biomass between PA23 exposed plants and plants exposed to the *gacS* and *hcn* mutants, suggesting that HCN exerts no negative effects on plant growth. By including the *gacS* mutant, we were able to explore whether pathways that govern biocontrol also regulate PGP volatile production. Comparing the *gacS*- and PA23-exposed plants revealed no obvious difference in biomass. Therefore, it appears the pathways that regulate biocontrol may not be involved in production of PGP volatiles. A similar result was seen in *Arabidopsis* plants when exposed to volatiles produced by *P. fluorescens* SBW25 and a *gacS* mutant, in which case plants exposed to both bacteria were larger compared to controls (Cheng et al. 2016). However, it was also shown that volatile PGP effects were dependent on plant species. When tobacco plants were exposed to bacterial volatiles, the wild type SBW25 had an inhibitory effect on root growth compared to the *gacS* mutant (Cheng et al. 2016). Therefore, the signalling pathways in different plant species play a major role in the bacterial-induced PGP effect.

Induced systemic resistance (ISR) is a defense mechanism where non-pathogenic growth promoting bacteria prime the plant to develop an enhanced immune defense against pathogens (Choudhary, Prakash, and Johri 2007). Different from systemic acquired resistance (SAR), which is activated in response to pathogens, ISR employs distinct regulatory pathways dependent

on jasmonic acid and ethylene signalling in the plant (Choudhary et al. 2007). Application of exogenous PGP volatile compounds produced by *B. subtilis*, identified as 2,3-butanediol and acetoin, triggered ISR in *Arabidopsis* plants, resulting in increased resistance to *Erwinia carotovora* (Ryu et al. 2004). Using transgenic plants, it was shown that the PGP effect of *B. subtilis* volatiles was dependent on ethylene production in the plants (Ryu et al. 2004). Studying whether PA23 volatiles elicit ISR in lettuce will allow a better understanding of molecular mechanisms underlying PGP signalling in plants.

While the individual volatiles produced by PA23 were not specifically investigated for their PGP effects, we believe one compound may be involved. It was previously reported that PA23 produces three volatiles that contribute to fungal suppression, namely nonanal, 2-ethyl 1-hexanol and benzothiazole (Fernando et al. 2005). Benzothiazole has been shown to promote plant growth in cucumber plants (Šimonová, Henselová, and Zahradnik 2005); therefore, this volatile may play a role in PA23-mediated PGP.

In order to market PA23 as a biocontrol or PGP product, the cells must be prepared as a formulation that supports a long shelf life. A good formulation should maintain cell viability for at least one year of storage (Bashan et al. 2014). Furthermore, it is important to select formulation methods that benefit the consumer; making sure the product is easy to use, has low production costs and is environmentally friendly (Bashan et al. 2014). Studies have shown that storage at cooler temperatures supports a higher cell viability compared to room temperature, regardless of formulation type (liquid or dry) (Cabrefiga et al. 2014; Corrêa, Sutton, and Bettiol 2015). In the current study, two different PA23 formulations were investigated to observe changes in cell viability upon storage at 4^{o}C over the course of one year.

Liquid formulations are desirable because they are easy to use and produce (Bashan et al. 2014). There are liquid formulations on the market that have a shelf life of two years (XiteBio). However, obtaining a long shelf life with liquid formulations remains a challenge because the bacteria remain metabolically active, rendering them sensitive to desiccation. The additives selected for the liquid formulations were chosen for their nutritional content, water holding capacity or structural protection potential. Also, they do not pose an environmental risk. At 4^{o}C, the cell viability of all of the liquid formulations decreased significantly after one year of storage. Analyzing cell viability, none of the additives tested out performed another. However, the additives selected represent a small fraction of the protectants available; other additives may maintain a higher shelf life of PA23-based formulations.

Dry formulated cell products are easy to handle, transport and store (Cabrefiga et al. 2014). At 4^{o}C, lyophilized PA23 cells maintained a higher viability compared to the liquid formulations. Furthermore, PA23 cells cultured in LB media and freeze-dried with lactose performed the best. Cells cultured in M9^{+} media are considered to be "osmoadapted", which increases resiliency to desiccation during freeze drying and when applied to the phyllosphere (Cabrefiga et al. 2014). Standard cultured cells were grown in LB broth and freeze-dried with lactose. When osmoadapted *P. fluorescens* EPS62e cells were freeze-dried (with lactose) and stored for one year, cell viability was higher than standard cultured cells (Cabrefiga et al. 2014). However, the opposite trend was seen in dry formulation experiments with PA23. One explanation for this difference could be species related. *P. fluorescens* EPS62e cells have a much higher tolerance to salt (NaCl) amended media (0.5%) (Cabrefiga et al. 2014), compared to PA23 (0.3%). Therefore, PA23 may preserve better when grown in a higher nutrient media like LB.

One downside associated with dry formulations is the more complex methodology and machinery required to lyophilize cells. Importantly, the dry-formulated cell products exhibited decreased disease suppression in cotyledon infection assays with *L. maculans*. An explanation for this poor performance is that the cells were not yet metabolically active; cell metabolism is arrested when cells are dried. Applying *L. maculans* 1hr after bacterial inoculation may not allow sufficient time for bacterial cells to become fully active. The dry formulation method was developed from a study employing *P. fluorescens* EPS62e (Cabrefiga et al. 2014). While biocontrol was evaluated in a similar manner, wound infection with *Erwinia amylovora* was done 24hr after bacterial inoculation. In this case, the dry formulations showed significant biocontrol capabilities on pear flowers (Cabrefiga et al. 2014). It is possible that increasing the PA23 incubation time prior to *L. maculans* infection will enable disease prevention by allowing cell metabolism to be fully restored.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Biotechnology with PGPR has become an important aspect in the agricultural industry in an effort to improve the sustainability of major Canadian crops like canola. Bacteria can promote plant growth in several ways, including defending the plant against pathogens, increasing nutrient availability, and producing growth hormones and enzymes that degrade plant stress signals (Glick 2012). *P. chlororaphis* strain PA23 exhibits antifungal activity against *S. sclerotiorum,* protecting canola plants from disease in greenhouse and fields experiments (Fernando et al. 2007; Savchuk and Fernando 2004; Zhang 2004). When applied as a combination of root dip and drench irrigation, significant increases in PA23-mediated lettuce biomass were observed (Nandi and de Kievit, unpublished results). These findings indicate that in addition to functioning as a biocontrol agent, PA23 may have PGP properties. Root dip application of bioinoculants is not a practical means of treating canola and lettuce for increased agricultural production. Therefore, the first objective of this study was to evaluate the PGP potential of PA23 on canola and lettuce seedlings when applied by drench irrigation. Trials were undertaken exploring the effect of bacterial concentration, times of treatment, and varying doses of bioinoculant application. Collectively there were no significant increases in plant biomass resulting from treatment with the PA23 wt. The *gacS* mutant which does not secrete exoproducts, also showed no PGP effects.

Many factors affect the PGP potential of bacteria, for example IAA, a signalling hormone produced by plants and some PGPR, is involved in virtually all plant growth and development processes (Glick 2012). Studies have shown that bacteria with the ability to produce IAA increase plant biomass (Patten and Glick 2002). However, a decreased PGP effect has been demonstrated in plants under stress; IAA signal transduction is inhibited by ethylene production

(Czarny *et al.*, 2006; Glick *et al.*, 2007; Stearns *et al.*, 2012). Ethylene, produced by plants under stress, acts as a signalling hormone that negatively impacts plant developmental processes, such as root elongation, fruit ripening and plant responses that promote beneficial interactions with PGPR (Glick 2012). To counteract this effect, some PGPR produce ACC deaminase, an enzyme that degrades the ACC precursor in the ethylene biosynthesis pathway preventing a stress response (Glick 2012). Therefore, ACC deaminase plays an important role in PGP by inhibiting ethylene production enabling IAA signal transduction to promote growth. Previously, studies have established that PA23 produces IAA but not ACC deaminase (Reimer, 2016). Gnotobiotic experiments that support more controlled conditions showed PA23 application increased root length on canola seedlings (Reimer, 2016). However, this PGP effect was not observed in soil experiments (Reimer, 2016). It is possible that IAA signal transduction may be more vulnerable to ethylene levels in soil environments. In a previous study, transfer of the ACC deaminase gene into *P. protegens* strain CHA0, which lacks ACC deaminase activity, resulted in increased root length compared to CHA0-treated plants (Wang et al. 2000). Future experiments should investigate whether PA23 expressing a gene encoding ACC deaminase results in PGP in soil-grown plants.

Organic volatiles produced by PGPR may exhibit both antifungal and PGP activity (Blom et al. 2011; Fernando et al. 2005). PA23 produces three volatile compounds that are suppressive to *S. sclerotiorum* (Fernando et al. 2005); however, growth promoting volatiles have not been explored. Therefore, the second objective of this thesis was to investigate the PGP potential of organic volatiles produced by PA23. Lettuce seedlings were exposed to PA23 and the *gacS* and *hcn* mutants for two weeks in an enclosed system. We discovered that all three strains produce volatiles that increase plant biomass. Through the use of the *gacS* mutant, which exhibits no

antifungal activity, we demonstrated that the pathways governing biocontrol do not regulate PGP volatile production. Furthermore, plant assays with the *hcn* mutant show that after two weeks of growth, HCN does not negatively impact plant health. In a study examining PGP effects of volatiles produced by *P. fluorescens* SS101, three PGP volatiles were identified using GC-MS (Park *et al.*, 2015). When synthetic forms of these volatiles were tested in plate assays, maximal PGP was induced at different concentrations depending on the compound (Park et al. 2015). In the future, synthetic forms of the three primary volatiles produced by PA23 should be tested for their PGP potential. These experiments may enable us to identify the volatile(s) responsible for PGP as well as the concentration required for maximal plant yield. To further understand the growth promotion seen in lettuce plants from exposure to PA23 volatiles, the signalling pathways involved with ISR and the interaction with bacterial volatiles should be investigated. Transcriptional analysis can be conducted to observe differences in expression of genes involved in ISR and plant growth, such as *SCYL2A/B* which are vital for cell growth and root hair development.

To commercialize a PGPR, a formulation must be developed that will preserve cells for long periods of time. When selecting a formulation, it is important to take into consideration production costs, user friendliness and the environmental risks of any incorporated additives (Bashan et al. 2014). In addition, one must keep in mind the method of application. Either a liquid or dry PA23-based formulation can be used as a foliar spray for control of *S. sclerotiorum* in the phyllosphere or as a drench application for PGP. Therefore, the third objective of this research was to evaluate the viability of PA23 in both dry and liquid formulations, over the course of one-year storage at 4^{o}C. The liquid formulations contained PA23 cells that were cultured and re-suspended in fresh LB with one or more of seven different additives reported to

protect against desiccation (Namsena, Bussaman, and Rattanasena 2016; Segarra et al. 2015) The dry formulations contained PA23 cells that were cultured in either nutritional media, minimal media or high salt amended minimal media. Growing bacterial cells in a salt amended minimal medium is reported to encourage osmoadaptation, increasing salt tolerance and making cells more resilient during freeze drying and storage (Cabrefiga et al. 2014). Furthermore, lactose was added to concentrated overnight cultures to protect cells during the freeze-drying process (Cabrefiga et al. 2014). After one year of storage at 4^{o}C, the viability of all the liquid formulations declined rapidly at a relatively similar rate. The dry formulations maintained a higher storage viability overall compared to the liquid formulations. Osmoadapted PA23 cells did not appear to have a drastic effect on viability after lyophilization and during storage. The dry formulated PA23 cells cultured in LB and lyophilized with lactose maintained the highest viability after storage at 4^{o}C compared to cells cultured in TB, M9 and M9^{+} media and lyophilized with lactose. However, testing the biocontrol efficacy of each of the formulations revealed that optimal viability does not correlate with maximum disease suppression. In infection assays with *L. maculans*, almost all of the liquid formulations prevented infection on cotyledons. Conversely, plants treated with the dry formulations succumbed to infection. For these assays, fungal spores were applied to cotyledon wounds one hour after bacterial inoculation. Most likely, this was not enough time for bacterial cells in the dry formulations to become metabolically active and produce the compounds necessary for fungal antagonism. It is recommended that infection assays be repeated with a longer bacterial incubation time on the cotyledons prior to fungal infection. This will allow us to better evaluate the efficacy of the dry preparation and ultimately determine which formulation is superior for storage viability and biocontrol.

REFERENCES

Agriculture and Agri-food Canada. 2018. “Status report for biopesticide projects and submissions”. *Government of Canada*. http://www.agr.gc.ca/eng/?id=1392136770253.

Agriculture and Agri-Food Canada. 2018. “We grow a lot more than you think.”*Government of Canada*. http://www.agr.gc.ca/eng/about-us/publications/we-grow-a-lot-more-than-you-may-think/?id=1251899760841.

Ahemad, Munees and Mulugeta Kibret. 2014. “Mechanisms and Applications of Plant Growth Promoting Rhizobacteria: Current Perspective.” *Journal of King Saud University - Science* 26(1):1–20. Retrieved (http://dx.doi.org/10.1016/j.jksus.2013.05.001).

Asari, Shashidar, Staffan Matzén, Mikael Agerlin Petersen, Sarosh Bejai, and Johan Meijer. 2016. “Multiple Effects of *Bacillus amyloliquefaciens* Volatile Compounds: Plant Growth Promotion and Growth Inhibition of Phytopathogens.” *FEMS Microbiology Ecology* 92(6):1–11.

Bashan, Yoav, Luz E. de-Bashan, S. R. Prabhu, and Juan Pablo Hernandez. 2014. “Advances in Plant Growth-Promoting Bacterial Inoculant Technology: Formulations and Practical Perspectives (1998-2013).” *Plant and Soil* 378(1–2):1–33.

Blom, D. et al. 2011. “Production of Plant Growth Modulating Volatiles Is Widespread among Rhizosphere Bacteria and Strongly.” 13:3047–58.

Bottini, Rubén, Fabricio Cassán, and Patricia Piccoli. 2004. “Gibberellin Production by Bacteria and Its Involvement in Plant Growth Promotion and Yield Increase.” *Applied Microbiology and Biotechnology* 65(5):497–503.

Cabrefiga, J., J. Francés, E. Montesinos, and A. Bonaterra. 2014. “Improvement of a Dry Formulation of *Pseudomonas fluorescens* EPS62e for Fire Blight Disease Biocontrol by Combination of Culture Osmoadaptation with a Freeze-Drying Lyoprotectant.” *Journal of Applied Microbiology* 117(4):1122–31. Retrieved (http://www.ncbi.nlm.nih.gov/pubmed/24947806).

Cheng, Xu. Viviane Cordovez. Desalegn W. Etalo. Menno van der Voort and Jos M. Raajimakers. 2016. "Role of the GacS sensor kinase in the regulation of volatile production by plant growth-promoting *Pseudomonas fluorescens* SBW25.” *Front Plant Sci.* 7 (1706): 1-15.

Choudhary, Devendra K., Anil Prakash, and B. N. Johri. 2007. “Induced Systemic Resistance (ISR) in Plants: Mechanism of Action.” *Indian Journal of Microbiology* 47(4):289–97.

Cipriano, Matheus A. P. et al. 2016. “Lettuce and Rhizosphere Microbiome Responses to Growth Promoting *Pseudomonas* Species under Field Conditions.” *FEMS Microbiology Ecology* 92(12):fiw197. Retrieved (https://academic.oup.com/femsec/article-lookup/doi/10.1093/femsec/fiw197).

Corrêa, E. B., J. C. Sutton, and W. Bettiol. 2015. “Formulation of *Pseudomonas chlororaphis* Strains for Improved Shelf Life.” *Biological Control* 80(January):50–55. Retrieved (http://dx.doi.org/10.1016/j.biocontrol.2014.09.009).

Devi, Karunanidhi Kanchana, Sharma Deepika, Asani Bhaduri, and David Kothamasi. 2013. “Polymorphism in hcnAB Gene in *Pseudomonas* Species Reveals Ecologically Distinct Hydrogen Cyanide-Producing Populations.” *Geomicrobiology Journal* 30(2):131–39.

Devi, Karunanidhi Kanchana and David Kothamasi. 2009. “Pseudomonas Fluorescens CHA0 Can Kill Subterranean Termite Odontotermes Obesus by Inhibiting Cytochrome c Oxidase of the Termite Respiratory Chain.” *FEMS Microbiology Letters* 300(2):195–200.

Duca, Daiana, Janet Lorv, Cheryl L. Patten, David Rose, and Bernard R. Glick. 2014. “Indole-3-Acetic Acid in Plant–microbe Interactions.” *Antonie van Leeuwenhoek* 106(1):85–125. Retrieved (http://link.springer.com/10.1007/s10482-013-0095-y).

Fernando, W. G. D., S. Nakkeeran, Y. Zhang, and S. Savchuk. 2007. “Biological Control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* Species on Canola Petals.” *Crop Protection* 26(2):100–107.

Fernando, W. G.Dilantha, Rajesh Ramarathnam, Akkanas S. Krishnamoorthy, and Sarah C. Savchuk. 2005. “Identification and Use of Potential Bacterial Organic Antifungal Volatiles in Biocontrol.” *Soil Biology and Biochemistry* 37(5):955–64.

Ghorbani, Reza, S. Wilcockson, A. Koocheki, and C. Leifert. 2008. “Soil Management for Sustainable Crop Disease Control: A Review.” *Environmental Chemistry Letters* 6(3):149–62.

Glick, Bernard R. 2012. “Plant Growth-Promoting Bacteria: Mechanisms and Applications.” *Scientifica* 2012:1–15. Retrieved (http://www.hindawi.com/journals/scientifica/2012/963401/).

Glick, Bernard R., Changping Liu, Sibdas Ghosh, and Erwin B. Dumbroff. 1997. “Early Development of Canola Seedlings in the Presence of the Plant Growth-Promoting Rhizobacterium *Pseudomonas putida* GR12-2.” *Soil Biology and Biochemistry* 29(8):1233–39.

Guil, J. L., I. Rodríguez-García, and E. Torija. 1997. “Nutritional and Toxic Factors in Selected Wild Edible Plants.” *Plant Foods for Human Nutrition* 51(2):99–107.

Gururani, Mayank Anand et al. 2012. “Plant Disease Resistance Genes: Current Status and Future Directions.” *Physiological and Molecular Plant Pathology* 78:51–65. Retrieved (http://dx.doi.org/10.1016/j.pmpp.2012.01.002).

Hassan, H. M. and I. Fridovich. 1980. “Mechanism of Action of Pyocyanine.” *Journal of Bacteriology* 141(1):156–63.

Hegedus, Dwayne D. and S.Roger Rimmer. 2005. “*Sclerotinia sclerotiorum*: When ‘to Be or Not to Be’ a Pathogen?” *FEMS Microbiology Letters* 251(2):177–84.

Kavitha, Kumaresan et al. 2005. “Broad Spectrum Action of Phenazine against Active and Dormant Structures of Fungal Pathogens and Root Knot Nematode.” *Archives of Phytopathology and Plant Protection* 38(1):69–76.

de Kievit, Teresa R., Bee Hameeda, Carrie Selin, and W. G. D. Fernando. 2011. “Using Molecular Techniques to Understand and Enhance Biological Control by *Pseudomonas* Spp.” *Bashanfoundation.Org*. Retrieved (http://www.bashanfoundation.org/dilantha/dilanmoleculartech.pdf).

Lapouge, Karine, Mario Schubert, Frédéric H. T. Allain, and Dieter Haas. 2008. “Gac/Rsm Signal Transduction Pathway of γ-Proteobacteria: From RNA Recognition to Regulation of Social Behaviour.” *Molecular Microbiology* 67(2):241–53.

Manikandan, R., D. Saravanakumar, L. Rajendran, T. Raguchander, and R. Samiyappan. 2010. “Standardization of Liquid Formulation of *Pseudomonas fluorescens* Pf1 for Its Efficacy against Fusarium Wilt of Tomato.” *Biological Control* 54(2):83–89. Retrieved (http://dx.doi.org/10.1016/j.biocontrol.2010.04.004).

Manuel, Jerrylynn, Carrie Selin, W. G. Dilantha Fernando, and Teresa de Kievi. 2012. “Stringent Response Mutants of *Pseudomonas chlororaphis* PA23 Exhibit Enhanced Antifungal Activity against Sclerotinia Sclerotiorum in Vitro.” *Microbiology* 158(1):207–16.

Mavrodi, Dmitri V., Wulf Blankenfeldt, and Linda S. Thomashow. 2006. “Phenazine Compounds in Fluorescent *Pseudomonas* Spp. Biosynthesis and Regulation.” *Annual Review of Phytopathology* 44(1):417–45. Retrieved (http://www.annualreviews.org/doi/10.1146/annurev.phyto.44.013106.145710).

Mishra, Jitendra and Naveen Kumar Arora. 2017. “Secondary Metabolites of Fluorescent Pseudomonads in Biocontrol of Phytopathogens for Sustainable Agriculture.” *Applied Soil Ecology* (May):0–1. Retrieved (http://linkinghub.elsevier.com/retrieve/pii/S0929139317306017).

Namsena, Piyarat, Prapassorn Bussaman, and Paweena Rattanasena. 2016. “Bioformulation of *Xenorhabdus stockiae* PB09 for Controlling Mushroom Mite, Luciaphorus Perniciosus Rack.” *Bioresources and Bioprocessing*.

Nandi, Munmun, Carrie Selin, Gabriel Brawerman, W. G.Dilanth. Fernando, and Teresa R. de Kievit. 2016. “The Global Regulator ANR Is Essential for *Pseudomonas chlororaphis* Strain PA23 Biocontrol.” *Microbiology (United Kingdom)* 162(12):2159–69.

Ortíz-Castro, Randy, Eduardo Valencia-Cantero, and José López-Bucio. 2008. “Plant Growth Promotion by *Bacillus megaterium* Involves Cytokinin Signaling.” *Plant Signaling & Behavior* 3(April):263–65.

Park, Yong Soon, Swarnalee Dutta, Mina Ann, Jos M. Raaijmakers, and Kyungseok Park. 2015. “Promotion of Plant Growth by *Pseudomonas fluorescens* Strain SS101 via Novel Volatile Organic Compounds.” *Biochemical and Biophysical Research Communications* 461(2):361–65. Retrieved (http://dx.doi.org/10.1016/j.bbrc.2015.04.039).

Patten, Cheryl L. and Bernard R. Glick. 2002. “Role of Pseudomonas Putida Indoleacetic Acid in Development of the Host Plant Root System.” *Applied and Environmental Microbiology* 68(8):3795–3801. Retrieved (http://aem.asm.org/cgi/content/abstract/68/8/3795%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=124051&tool=pmcentrez&rendertype=abstract).

Pérez-Montaño, F. et al. 2014. “Plant Growth Promotion in Cereal and Leguminous Agricultural Important Plants: From Microorganism Capacities to Crop Production.” *Microbiological Research* 169(5–6):325–36.

Peter C. Loewen, Jacylyn Villenueva, W. G. Dilantha Fernando, Teresa de Kievit et al. 2014. “Genome Sequence of *Pseudomonas chlororaphis* Strain PA23.” *Genome Announcements* 2(4). Retrieved (http://genomea.asm.org/lookup/doi/10.1128/genomeA.00581-16).

Pillonel, Christian and Thomas Me. 1997. “Effect of Phen Y Lp Y Rroles on Gl Y Cerol Accumulation and Protein Kinase Activit Y of Neurospora Crassa.” .

Poritsanos, N., C. Selin, W. G.Dilantha Fernando, S. Nakkeeran, and Teresa R. De Kievit. 2006. “A GacS Deficiency Does Not Affect *Pseudomonas chlororaphis* PA23 Fitness When Growing on Canola, in Aged Batch Culture or as a Biofilm.” *Canadian Journal of Microbiology* 52(2006):1177–88.

Ryu, C. M. Mohamed A. Farag. Chia-Hui Hu. Munagala S. Reddy. Joseph W. Kloepper and Paul W. Paré. 2004. “Bacterial Volatiles Induce Systemic Resistance in Arabidopsis.” *Plant Physiology* 134(3):1017–26. Retrieved (http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.026583).

Savchuk, Sarah and W. G.Dilanth. Fernando. 2004. “Effect of Timing of Application and Population Dynamics on the Degree of Biological Control of *Sclerotinia sclerotiorum* by Bacterial Antagonists.” *FEMS Microbiology Ecology* 49(3):379–88.

Segarra, Guillem, Gerardo Puopolo, Oscar Giovannini, and Ilaria Pertot. 2015. “Stepwise Flow Diagram for the Development of Formulations of Non Spore-Forming Bacteria against Foliar Pathogens: The Case of Lysobacter Capsici AZ78.” *Journal of Biotechnology* 216:56–64. Retrieved (http://dx.doi.org/10.1016/j.jbiotec.2015.10.004).

Selin, Carrie et al. 2010. “Phenazines Are Not Essential for *Pseudomonas chlororaphis* PA23 Biocontrol of *Sclerotinia sclerotiorum*, but Do Play a Role in Biofilm Formation.” *FEMS Microbiology Ecology* 71(1):73–83.

Selin, Carrie, W. G.Dilanth. Fernando, and Teresa de Kievit. 2012. “The PhzI/PhzR Quorum-Sensing System Is Required for Pyrrolnitrin and Phenazine Production, and Exhibits Cross-Regulation with RpoS in *Pseudomonas chlororaphis* PA23.” *Microbiology* 158(4):896–907.

Selin, Carrie, Jerrylynn Manuel, W. G.Dilantha Fernando, and Teresa De Kievit. 2014. “Expression of the *Pseudomonas chlororaphis* Strain PA23 Rsm System Is under Control of GacA, RpoS, PsrA, Quorum Sensing and the Stringent Response.” *Biological Control* 69:24–33. Retrieved (http://dx.doi.org/10.1016/j.biocontrol.2013.10.015).

Šimonová, E., M. Henselová, and P. Zahradnik. 2005. “Benzothiazole Derivatives Substituted in Position 2 as Biologically Active Substances with Plant Growth Regulation Activity.” *Plant, Soil and Environment* 51(11):496–505.

Spaepen, Stijn, Jos Vanderleyden, and Roseline Remans. 2018. “Indole-3-Acetic Acid in Microbial and Microorganism-Plant Signaling.” (April):425–48.

Wang, Chunxia, Edouard Knill, Bernard R. Glick, and Geneviève Défago. 2000. “Effect of Transferring 1-Aminocyclopropane-1- Carboxylic Acid (ACC) Deaminase Genes into Pseudomonas Fluorescens Strain CHA0 and Its Gac A Derivative CHA96 on Their Growth-Promoting and Disease-Suppressive Capacities.” 907:898–907.

Zhang, Y. et al. 2006. “Detection of Antibiotic-Related Genes from Bacterial Biocontrol Agents with Polymerase Chain Reaction.” *Canadian Journal of Microbiology* 52(5):476–81. Retrieved (http://www.nrcresearchpress.com/doi/abs/10.1139/w05-152).