MANAGEMENT OF FUSARIUM BASAL ROT DISEASE OF ONION (*ALLIUM CEPA* L.) BY USING PLANT GROWTH PROMOTING RHIZOBACTERIA IN SEAWEED FORMULATION

by

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ABSTRACT

Onion (*Allium cepa* L.) is one of the most important vegetable crops cultivated around the world. The production of onion is negatively affected by fungal and bacterial diseases. *Ascophyllum nodosum* extract (ANE) could increase plants' resistance to pathogenic fungi and bacteria. Plant growth-promoting rhizobacteria (PGPR) have been widely used as effective biocontrol agents. The present study explored the efficiency of three PGPR strains (*Pseudomonas protegens* CHA0, *Bacillus subtilis*, and *Enterobacter cloacae* CAL2), ANE, and the combination of ANE and each PGPR strain in controlling onion Fusarium basal rot disease caused by *Fusarium oxysporum* f. sp. *cepae* (FOC). Under *in vitro* conditions, all PGPR strains showed antagonist activity against FOC, especially *E. cloacae* CAL2 displayed the highest antagonistic activity. Under *in vivo* conditions, *E. cloacae* CAL2, ANE, and their combined applications had no positive effects on onion biomass in the presence/absence of pathogen challenge, and 0.5% ANE had a negative effect on the biomass parameters (shoot and root fresh weight) of onion plants. All the treatments provided no control of the relative biomass of FOC in onion root tissues, especially ANE application resulted in the highest FOC biomass. Meanwhile, no significant changes in the activity of the defence-related enzymes and total phenolics and hydrogen peroxide contents were found. The results of the present study provide a basis for better exploring the combined application of PGPR and seaweed extract or other biocontrol agents to control plant diseases.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANE	*Ascophyllum nodosum* extract
$	Dollar
%	Percentage
°C	Degrees Celsius
µg	Microgram(s)
A. cepa	*Allium cepa*
A. fistulosum	*Allium fistulosum*
A. galanthum	*Allium galanthum*
A. giganteum	*Allium giganteum*
A. nodosum	*Ascophyllum nodosum*
A. schoenoprasum	*Allium schoenoprasum*
A. thaliana	*Arabidopsis thaliana*
ACC	1-aminocyclopropane-1-carboxylic acid
ANOVA	Analysis of Variance
B. subtilis	*Bacillus subtilis*
B. velezensis	*Bacillus velezensis*
BP	Years before present
bp	Base pair(s)
BS	*Bacillus subtilis*
BY-2	Bright Yellow-2
c.f.u	Colony-forming unit
CAL2	*Enterobacter cloacae* CAL2
CHA0	*Pesudomonas protegens* CHA0
cm	Centimetre(s)
CMC	Carboxyl methylcellulose
cpm	Counts per minute

CRD	Completely randomized design
Ct	Cycle threshold
DFE	Dietary Folate Equivalents
DNA	Deoxyribonucleic acid
E. cloacae CAL2	*Enterobacter cloacae* CAL2
endo-PTE	Endo-pectintrans-eliminase
exo-PG	Exo-polygalacturonase
FBR	Fusarium basal rot
FOC	*Fusarium oxysporum* f. sp. *Cepae*
FW	Fresh weight
g	Gram(s)
h	Hour(s) (unit of time)
H_2O_2	Hydrogen peroxide
HCN	Hydrogen cyanide
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
JA	Jasmonic acid
kb	Kilo base pairs
Kcal	Kilocalorie
KMA	King's B agar
L	Liter(s)
M	molar, mol/L
mg	Milligram(s)
min	Minute(s) (unit of time)
ml	Millilitre(s)
mm	Millimetre

mM	Millimolar
Na2CO3	Sodium carbonate
NaOCl	Sodium hypochlorite
ng	Nanogram
nm	Nanometre(s)
NS	Nova scotia
OD	Optical density
P	P value
P. aeruginosa	*Pseudomonas aeruginosa*
P. protegens CHA0	*Pesudomonas protegens* CHA0
PAL	Phenylalanine ammonia lyase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pg	Picogram(s)
PGPR	Plant growth promoting rhizobacteria
pH	Potential of hydrogen
PO	Peroxidase
ppm	Parts per million
PPO	Polyphenol oxidase
PR	Pathogenesis-related
RAE	Retinol Activity Equivalents
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Second(s) (unit of time)
SA	Salicylic acid
SAR	Systemic acquired resistance

SDW	Sterilized distilled water
SE	Standard Error
T. asperellum	*Trichoderma asperellum*
T. virens	*Trichoderma virens*
TPC	Total phenolic content
TSA	Trypticase soy agar
w/v	weight/volume
α	Significance level
µl	Microlitre(s)
µm	Micrometre

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CHAPTER 1 : GENERAL INTRODUCTION

1.1 INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important vegetable crops in the world. The production of onion in Canada is around 256 thousand tonnes, with a market value of $115 million (Statistics Canada, 2020). Onion has considerable commercial importance due to its edible and medical values. Onion has a strong characteristic taste and smell and can be cooked in many different ways. It also contains many bioactive compounds, such as flavonoids and organosulphur compounds, which have potential antioxidant, anti-inflammatory, anticancer (Mertens-Talcott & Percival, 2005; Iranshahi et al., 2015), antiplatelet (Goldman et al., 1996), and anti-obesity (Moon et al., 2013) activities.

The production of onion is generally affected by diseases caused by fungi and bacteria. The Fusarium basal rot (FBR) of onion, which is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cepae* (FOC), is one of the most devastating diseases of onion in the world (Taylor et al., 2019). The disease infection can occur at any growth stage of the onion plant, causing severe damage both in the field and storage (Cramer, 2000; Taylor et al., 2019). Several methods have been proposed for the management of the disease, such as crop rotation (Leoni et al., 2013), the use of resistant cultivars (Cramer, 2000; Cramer et al., 2021; Karaca et al., 2012), and chemical fungicides applications (Degani & Kalman, 2021). However, none of these methods can provide effective control of FBR. Therefore, developing a new eco-friendly and viable approach for controlling onion FBR disease is an urgent need.

Biological control of plant disease has been receiving more and more attention. Seaweeds and their extracts have been used in agricultural systems for a long time due to their beneficial effects on crop growth and productivity (Craigie, 2011; Khan et al., 2009). The extract of *Ascophyllum nodosum*, a brown seaweed, has been shown to induce plant resistance to pathogenic fungi and bacteria (Shukla et al., 2019). Research has shown that the application of *Ascophyllum nodosum* extract reduced fungal disease in carrot and cucumber plants (Jayaraj et al., 2008; Jayaraman et al., 2011). Plant growth-promoting rhizobacteria (PGPR) is a group of bacteria that are able to colonize plant roots and improve plant growth by a wide variety of mechanisms, such as nitrogen fixation, phytohormones production, siderophore production, and induction of systemic resistance (Basu et al., 2021; Goswami et al., 2016; Vejan et al., 2016). Many studies have reported different PGPRs (e.g., *Pseudomonas* spp., *Bacillus* spp., and *Enterobacter* spp.) showed biocontrol effects in many crops (Ghadamgahi et al., 2022; Khan et al., 2018; Mohamed et al., 2020) and some of PGPR strains already had been commercialized, such as Nodulator LQ (*Bradyrhizobium japonicum*) and Serenade SOIL (QST 713 strain of *Bacillus subtilis*).

Therefore, it is thought that ANE, PGPR, or their combination have the potential to manage FBR disease on onion.

1.2 HYPOTHESIS

The hypothesis for this study was that the combined application of ANE and PGPR would be the most effective in controlling onion FBR disease.

1.3 OBJECTIVES

• To investigate the antagonistic activity of ANE and PGPR alone or in combination against FOC under *in vitro* conditions.

• To investigate the effect of ANE and PGPR on the control of onion FBR disease under greenhouse conditions.

CHAPTER 2 : LITERATURE REVIEW

2.1 ONION

Onion (*Allium cepa* L.) is one of the most significant vegetable crops and is an important part of the global human diet due to its pungent flavour and beneficial compounds (Mota et al., 2010; Sharma et al., 2016). The onion belongs to the *Amaryllidaceae* family of *Allium* genus which includes over 800 species (Angiosperm Phylogeny Group, 2009; Fritsch et al., 2010; Li et al., 2010). It is a shallow-rooted, biennial monocot but is usually cultivated as an annual for the production of its bulbs and as a biennial for the production of its seeds because inflorescences are formed in the second year (Steentjes et al., 2020). A full-grown onion plant has an inflorescence, leaves, a bulb, and fibrous roots (Figure 1). The bulb is mainly composed of bud(s), a modified compact stem surrounded by fleshy modified scale leaves, and a tunic that protects the bulb (Figure 2). The main edible part of the onion is the bulb, with a distinctive flavor and pungent smell. The bulbs usually have three colors (red, yellow/brown, and white) with sweet and non-sweet flavours (Albishi et al., 2013; Shahidi & Naczk, 2003). The formation of the onion bulb is affected by environmental factors in the growing regions, especially the day length (Cardoso & Costa, 2003). Onion varieties are categorized into long-day, intermediate-day, and short-day according to the minimum daily duration of light required for bulb forming (Gökçe et al., 2010). Onion seeds and transplants are usually used by growers (Province of British Columbia, 2020). Fresh onion bulbs and their processed products, such as dried powered, flakes, and essential oil, can be supplied for consumption (Arslan & Musa Özcan, 2010; Khan et al., 2016; Ye et al., 2013).

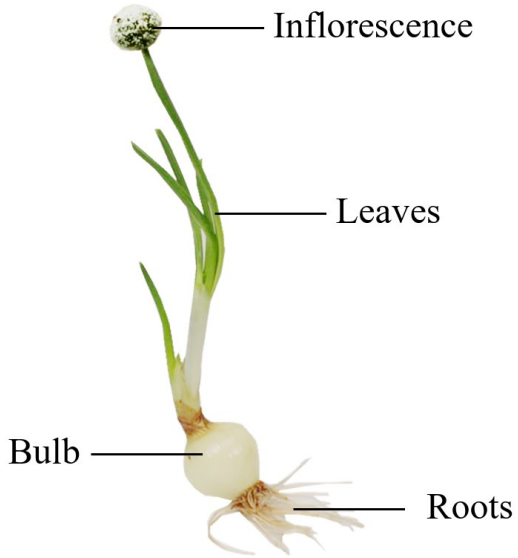


Figure 1. An onion plant with major anatomical features including inflorescence, leaves, bulb, and roots.

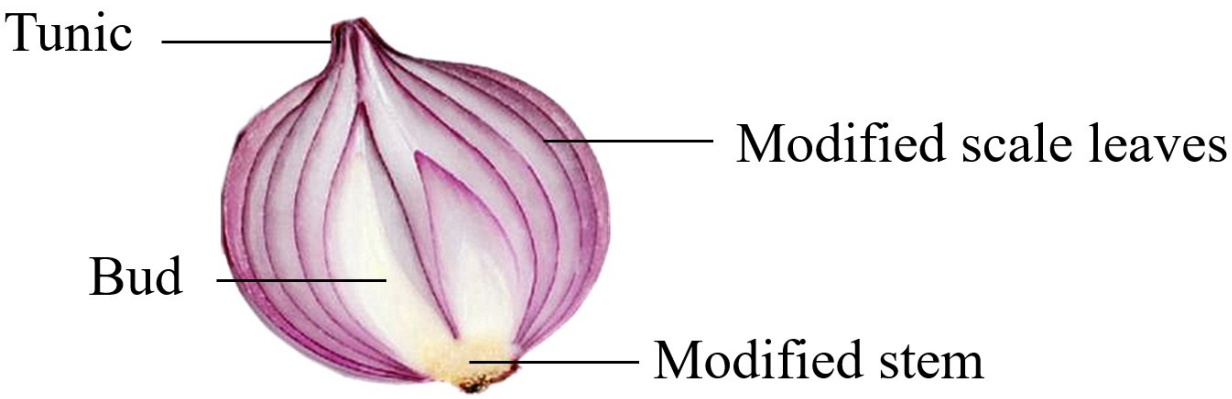


Figure 2. Longitudinal section of an onion bulb.

Onion is native to central Asia and has been cultivated for over 4000 years (Ren et al., 2019). It is now commercially cultivated in more than 170 countries around the world (Bahram-Parvar & Lim, 2018). It is one of the most important vegetables in the world, with a global planting area of about 5 million hectares and a total global production of 100 million tonnes; only tomatoes exceed bulb onion in importance based on global vegetable

production (FAOSTAT, 2020a). Asia took the lead in producing dry onion in 2020 (Figure 3). China (annual production of 25 million tons), India (annual production of 23 million tons), and the USA (annual production of 3 million tons) are the top three onion-producing countries in the world (FAOSTAT, 2020b). In Canada, the total production of onion was around 256 thousand tons, with a market estimated at $115 million in 2020 (Statistics Canada, 2020). The four top-producing provinces (Figure 4) were Quebec (41.8%), Ontario (37.9%), and Alberta (11.1%), followed by Nova Scotia (4.34%).

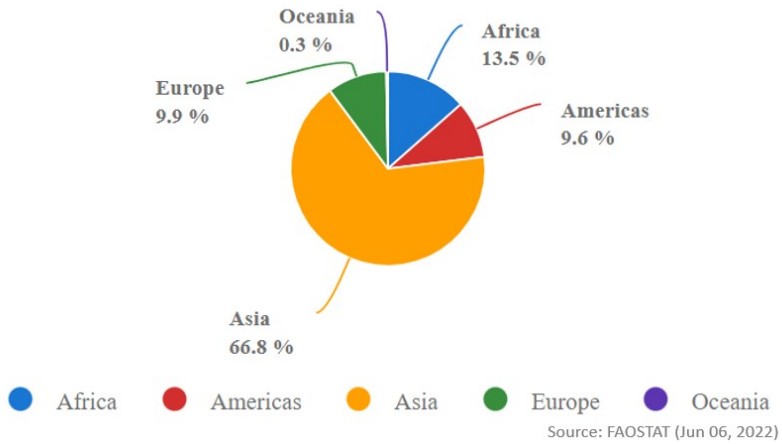


Figure 3. Production of onion (dry) by region – percent share (adapted from FAOSTAT, 2020b).

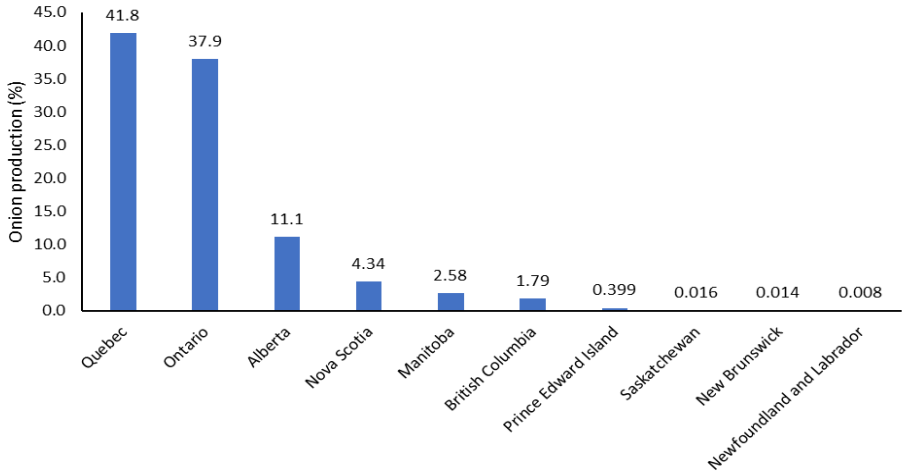


Figure 4. Fresh Dry Onion production in Canada (adapted from Statistics Canada, 2020).

People enjoy the numerous culinary uses of the onion. It can be eaten fried, cooked, pickled, or raw in many dishes, such as salads, soups, and stews (Van Wyk, 2014). Apart from its edible properties, onion also has many health benefits due to its nutritional composition (Table 1). It is a good source of protein, sugar, vitamins, minerals, flavonoids, amino acids, and other nutrients (Kwak et al., 2017; Sharma et al., 2016). Among these, the flavonoids and organosulphur compounds are the most important compounds that have potential antioxidant, anti-inflammatory, anticancer (Iranshahi et al., 2015; Mertens-Talcott & Percival, 2005), antiplatelet (Goldman et al., 1996), and anti-obesity (Moon et al., 2013) properties. Studies have reported that bioactive ingredients in onions have the potential to prevent cancer (Srivastava et al., 2016), inflammation (Seo et al., 2019), hyperglycemia (Jiang et al., 2019), and cardiovascular diseases (Suleria et al., 2015).

Table 1. Nutritional composition of onion (Health Canada, 2005).

Nutrients	Weight (41 g of onion)	
Energy	17	kcal
Protein	trace	g
Carbohydrate	4	g
Total Sugar	2	g
Total Dietary Fibre	0.6	g
Total Fat	trace	g
Calcium	9	mg
Iron	0.1	mg
Sodium	1	mg
Potassium	58	mg
Magnesium	4	mg
phosphorus	11	mg
Vitamin A	0	RAE
Beta-carotene	trace	mcg
Lycopene	0	mcg
Folate	8	DFE
Vitamin C	3	mg
Vitamin B12	0	mcg

2.2 MAJOR ONION DISEASES

Diseases are one of the major problems in onion production. Onion production and quality are damaged by many foliar, bulb, and root fungal and bacterial pathogens. The common diseases of onion are listed in Table 2 with their causal agent, effects, and suitable climatic conditions.

Table 2. Major onion fungal and bacterial diseases with the causal agent, effects, and suitable climatic conditions.

Disease	Pathogen	Effects	Suitable climatic conditions
Botrytis blight	*Botrytis squamosa*	The leaf infection causes leaf tips to die back and eventually leads to leaf death and small bulbs.	Moderate temperature, humid conditions
Purple blotch	*Alternaria porri*	The leaf infection results in leaf death and bulb rot.	Wet, humid conditions
Neck rot	*Botrytis allii*	Most commonly occurs on stored onions. The symptoms start around the neck of the bulb, causing the bulb diseased tissue collapses and becomes soft and spongy.	Moderate temperatures with high humidity
Fusarium basal rot	*Fusarium oxysporum*	The fungus infects roots and bulbs, causing plant wilt and rotting of roots and bulbs. Infected onions may not show symptoms in the field but may rot in storage.	High soil temperature (25 - 28℃) and moisture
Downy mildew	*Peronospora destructor*	The fungus attack onion leaves and causes the collapse of the leaf tissue. Bulbs can also be infected, resulting in storage rot.	cool weather with high humidity (> 95%)
Pink root	*Phoma terrestris*	Infected roots turn to pink, red, then purple brown. Root mass can be reduced by the fungal infection, causing undersized bulbs.	Warm (24-28 ℃) conditions

Table 2. Major onion fungal and bacterial diseases with the causal agent, effects, and suitable climatic conditions.

Disease	Pathogen	Effects	Suitable climatic conditions
White rot	*Sclerotium cepivorum*	leaves turn yellow, wilting and dropping. Roots and bulbs rot will be observed.	Cool (20 ℃) conditions
Soft rot	*Pectobacterium carotovorum subsp. carotovorum*	It occurs mainly on mature bulbs. As the disease progresses, infected bulb scales become soft and sticky, and eventually, the entire bulb rot and become soft and watery.	Warm wet weather in field. Above 3 ℃ in storage or transit

2.2.1 FUSARIUM BASAL ROT

The genus *Fusarium* comprises several phytopathogenic and toxigenic fungal species that are widely distributed (Ma et al., 2010; Parra-Rivero et al., 2020). *Fusarium oxysporum*, one of the most destructive soil-borne pathogens, is pathogenic to over 100 plant species (e.g., onion, tomato, cucumber, banana, and potato), causing many disease symptoms such as damping-off, root rots, or vascular wilt on plants (Abdelkhalek et al., 2020; Carmona et al., 2020; Cha et al., 2016; Fravel et al., 2003; Lastochkina et al., 2020; Mon et al., 2021; Sasaki et al., 2015). Onion FBR is a serious disease of onion caused by FOC. It can occur during both the pre-harvest growing and post-harvest storage periods (Cramer, 2000; Taylor et al., 2019).

Onion FBR disease has been reported to cause up to 90% loss of seedlings, 50% loss in the field, and 30-40% in the storage of onion crops (Gupta & Gupta, 2013; Mishra et al., 2014). In the Annapolis Valley of Nova Scotia, Canada, the increased disease incidence and severity of FBR has caused an estimated annual loss of up to $600,000 (or 20% of crop value) for onion growers (Bunbury-Blanchette & Walker, 2019). FBR caused 3% to 35% loss in yield and up to 75% of storage loss of onion in the USA (Abawi, 1972; Lacy, 1982;

Straley et al., 2021). The pathogen not only affects all parts of the onion plants but also affects other cultivated Allium species, such as shallots, Welsh onion, chives, and garlic (Cramer, 2000; Galván et al., 2008). It has been reported that FBR has an adverse effect on the conservation of *Allium* germplasms, as evidenced by the 20% loss of *A. giganteum* (an ornamental *Allium* species) bulbs at a germplasm resource center in Yunnan Province, China, in 2013 (Zhang et al., 2016). Crop rotation, use of resistant cultivars and seed treatment with chemical fungicides are the main methods that are applied to control FBR at present (Conn et al., 2012; Province of British Columbia, 2020).

FBR is caused by the pathogen FOC, belonging to phylum Ascomycota, class Sodariomycetes, and family *Nectriaceae*.

2.2.2 LIFE CYCLE

FOC forms three kinds of asexual spores: non-septate microconidia (2.5–15μm × 2–3μm), 3-septate macroconidia (15–20μm × 2.5–3μm), and chlamydospores (Cramer, 2000; Bektas & Kusek, 2019). Microconidia, oval-shaped to kidney-shaped, are abundant in the culture medium. Macroconidia are sickle-shaped, slightly tapered at both ends, and short to medium in length. Chlamydospores, abundantly formed in soil, are spherical and thick-walled. They are long-term survival structures that allow the pathogen to survive in the soil for several years, and they are also the main source of the primary inoculum of basal rot (Brayford, 1996; Burgess et al., 1988; Cramer, 2000). FOC overwinters as chlamydospores in soil and infected host plant residue. When soil temperatures and humidity are optimal, chlamydospores germinate and the germ tubes penetrate the host plant roots either directly or through natural openings/wounds. After penetration, the hyphae spread into the cortex and then invade xylem tissue. The pathogen will produce conidia and grow hyphae in the

xylem tissue, ultimately resulting in necrosis of infected tissues and death of the infected plants. The remaining infected plant debris in the soil starts the disease cycle again (Bragard et al., 2022; Cramer, 2000; Kant et al., 2011; Le et al., 2021b; Yadeta & Thomma, 2013).

FOC persists in soil for a long time as chlamydospores or as saprophytes on soil or infected plant residues. The optimum climatic conditions for disease development are high soil temperature (25°C-32°C) and moisture. However, the diseases can also occur when the soil temperature is between 15°C and 32°C. The disease rarely occurs when the soil temperatures are below 12°C (Crête et al., 1981; Cramer, 2000).

2.2.3 PATHOGENESIS

FOC is classified as a hemibiotrophic pathogen because it has a biotroph at the beginning of the infection but eventually changes to a necrotroph (Lyons et al., 2015). The pathogen can be spread in a variety of ways (e.g., infected plant debris, infected soil, farming irrigation water, farming equipment, onion seeds, and transplanted onion plants). In the biotrophic phase, FOC can recognize host plant roots through host-pathogen signals and attach to the surface of onion plants, such as basal plate, roots, and wounds. It produces hyphae and releases pectic enzymes, such as exo-polygalacturonase (exo-PG) and endo-pectin-trans-eliminase (endo-PTE), which can break the plant cell wall (Cramer, 2000). Therefore, the hyphae can grow through the cortex and enter the vascular system (phloem and xylem). The colonization and growth of fungal hyphae in various parts of the plant can cause the blockage of vessels (Srinivas et al., 2019). The vessel blockage disturbs water transport in plants and causes plant wilting (Di et al., 2016; Srinivas et al., 2019). As the

infection progresses, the pathogen changes to a necrotrophic lifestyle, causing leaf necrosis and decay and eventual plant death (Lyons et al., 2015).

2.2.4 SYMPTOMS

The initial above-ground symptoms of FBR on mature onion plants are chlorosis of all leaves. Then, the chlorosis develops into necrosis of the leaf tip and eventually leads to the necrosis of the entire leaf and plant death. The underground symptoms include root death and root abscission, red-brown discoloration, total necrosis of the basal plate, and bulb rot. The separation of roots and bulbs from the basal plate is a prominent symptom during uprooting. Healthy onion bulbs at the harvest stage also can be infected and then rot during storage (Cramer, 2000).

2.2.5 MANAGEMENT PRACTICES

FOC is a soil-borne pathogen that is difficult to manage because it can produce chlamydospores as long-lived survival structures. At present, cultural and chemical management methods are the main ways to manage onion FBR.

Cultural control methods include resistant cultivars and crop rotation. It has been found that some unpopular cultivated species (e.g., *Allium fistulosum*, *Allium schoenoprasum*, and *Allium galanthum*) are consistently resistant to FBR. The genetic resources of these resistant species can be used to develop resistance in onions against *Fusarium* spp. Although some studies reported resistance to FOC in some onion varieties (Table 3), there is no complete resistance has been found in *A. cepa*. Crop rotation with non-host crops for at least four years may reduce onion bulb losses to FBR (Seminis, 2020). Earlier studies have found that a sequence of crops (e.g., foxtail millet, wheat, and cowpea) can be

included in a rotation system to reduce the pathogen inoculum and limit onion FBR (Leoni et al., 2013). The combination of long-time crop rotation and onion cultivars for resistance to FBR is a more effective method (The Ontario Ministry of Agriculture, Food & Rural Affairs, 2016). However, the resistance is often broken down by new pathogenic races; meanwhile, the cultivation and development of new resistant cultivars are time-consuming because of the biennial generation time of the onion, and it can take up to 20 years to breed beneficial traits into a commercial cultivar (Cramer, 2000; Cramer et al., 2021; Karaca et al., 2012). Moreover, the use of resistant cultivars is restricted by the production areas and limited sources of naturally resistant cultivars (Rout et al., 2016).

Table 3. FBR-resistant onion varieties.

Onion varieties	Country	References
NuMex Dulce	USA	(Cramer, 2000)
NuMex Vado	USA	(Cramer, 2000)
NuMex Luna	USA	(Gutierrez & Cramer, 2004)
NMSU 00-32	USA	(Gutierrez & Cramer, 2004)
NMSU 00-13-1	USA	(Gutierrez & Cramer, 2004)
Rossa Savonese	Spain	(Galván et al., 2008)
NMSU 99-30	USA	(Gutierrez et al., 2006)
NuMex Arthur	USA	(Gutierrez et al., 2006)
NuMex Jose Fernandez	USA	(Gutierrez et al., 2006)
Ailsa Craig Prizewinner	UK	(Taylor et al., 2013)
White Lisbon	UK	(Taylor et al., 2013)
PI 256326 (Baia Periforme)	Brazil	(Mandal & Cramer, 2021)
PI 656956 (S015)	Syria	(Mandal & Cramer, 2021)

Currently, there are no fungicides registered for the control of FBR in Canada. Chemical seed treatments are widely used before planting to control FBR. Onion growers have been using Thiram, which acts as a seed protectant to prevent seed decay, seedling blight, and damping-off, to control FBR. No activity against FBR was detected from using Thiram. Meanwhile, Thiram is being deregistered. Therefore, F300, a mix of Maxim, Apron, and Dynasty, will be used by growers to replace Thiram in the near future (R. Madden, Personal communications, 2020). Furthermore, the application of chemicals can also bring many adverse impacts, such as the development of fungicide resistance in pathogens, the residues of pesticides in foods and the environment, and the death of non-target organisms. The management of plant disease needs a new eco-friendly and practical approach to reduce disease frequency and severity and minimize the use of pesticides. Biological control would be a possible solution to control FBR.

2.3 SEAWEED EXTRACT

Seaweed (macroalgae) is the general term that describes a group of macroscopic, multicellular marine algae (Salehi et al., 2019). Seaweeds are divided into three main categories based on their pigmentation (Khan et al., 2009), which are green algae (Chlorophyta), brown algae (Phaeophyta), and red algae (Rhodophyta). Seaweeds and their products have many applications in food, fuel, cosmetics, medical, and botanical areas (Ghaderiardakani et al., 2019). It was recently reported that the history of seaweeds as an important resource dates back to pre-historic times, around 14,000 BP (Dillehay et al., 2008). Seaweeds and their extracts have a long history of being used as a soil amendment to improve crop productivity in coastal agriculture. Growers traditionally use fresh or dried seaweed as fertilizer or compost (Craigie, 2011).

In order to meet the needs of the rapid global population growth, synthetic chemicals (fertilizers and pesticides) have been used excessively to increase agriculture and livestock production. At present, people are concerned about the adverse effects of the excessive use of synthetic chemicals on human health and the environment. Thus, seaweed extracts have been widely used to promote plant growth and protect plants. In the meantime, seaweed extracts can be applied as a soil application, seed treatment, and foliar spray (Mukherjee & Patel, 2020). Seaweed extracts consist of multiple components, such as carbohydrates (e.g., polysaccharides), minerals, plant growth hormones (e.g., auxins, cytokinins, ethylene, and gibberellins), proteins, amino acids, lipids, and vitamins (Nabti et al., 2017). Seaweed products can be used as bio-stimulants to enhance nutrient use efficiency, improve yield and nutritional quality of crops, alleviate abiotic stress, induce plant defence response against biotic stress, and improves soil health (EL Boukhari et al., 2020; Mukherjee & Patel, 2020).

The most commonly used seaweed in the production of agricultural biostimulants is brown algae (Phaeophyceae) (Craigie, 2011; Khan et al., 2009), such as *Ascophyllum nodosum*, *Ecklonia maxima*, and *Durvillea potatorum* (Sharma et al., 2014). *Ascophyllum nodosum* (L.) Le Jolis, or rockweed, is the most studied seaweed among brown algae. *A. nodosum* is abundant in the rocky intertidal zone of the North Atlantic coast of Canada and the estuarine rocky shores of Northern Europe (Khan et al., 2012). *A. nodosum* has become one of the most famous commercial seaweed crops in Canada. It is dominant in the intertidal zone along the coastline of the Maritimes in Atlantic Canada, where it forms extensive and dense beds (Pereira et al., 2020). Nova Scotia (mostly the western and

southern shores) harvests about 20,000 tons of rockweed per year. The harvest in the New Brunswick Bay of Fundy is about 10,000 tons per year (Government of Canada, 2015).

Currently, around 47 companies worldwide are involved in the production of *A. nodosum* extract for agricultural and horticultural purposes (Van Oosten et al., 2017). *A. nodosum* contains polysaccharides (e.g., alginates), storage carbohydrates (e.g., laminarin, mannitol, and fucans), plant growth hormones (e.g., auxins, cytokinins, and gibberellins), betaines, proteins, amino acids, lipids, and various minerals (e.g., calcium, magnesium, and potassium) as well as unidentified compounds with hormone-like activities (De Saeger et al., 2020; Khan et al., 2009; Sharma et al., 2014; Yakhin et al., 2017). In addition, it is also rich in phenols, such as polyphenols (fucol, fucophlorethol, fucodiphloroethol G, and ergosterol) and phenol group phlorotannin (Holdt & Kraan, 2011). *A. nodosum* has numerous beneficial effects in agriculture (Table 4), including improving plant growth and quality, increasing yield, mitigating abiotic stress in plants, improving plant defence against multiple pathogens, and improving soil health (De Saeger et al., 2020; Shukla et al., 2019).

Table 4. Effects of different *A. nodosum* extracts on various plants and soil systems.

S. No.	Extract	Crop	Function	Reference
1	*A. nodosum* based extract	Sweet cherry (*Prunus avium* L.)	Improved fruit quality and nutrient content of cherry	(Gonçalves et al., 2020)
2	Non-commercial *A. nodosum* extract	Grape (*Vitis vinifera*)	Improved eco-physiological parameters and affected secondary metabolism of grape	(Salvi et al., 2019)
3	Commercially available liquid *A. nodosum* extract	Maize (*Zea mays* L.)	Promoted root morphology traits and improved nutrition content of maize	(Ertani et al., 2018)

Table 4. Effects of different A. nodosum extracts on various plants and soil systems.

S. No.	Extract	Crop	Function	Reference
4	*A. nodosum* alkaline extract (Acadian Seaplants Limited)	Tobacco (*Nicotiana tabacum* L.)	Improved cell survival under cold stress	(Zamani-Babgohari et al., 2019)
5	*A. nodosum* extract (Acadian Seaplants Limited)	*Arabidopsis thaliana* (Col-0)	Modulated the expression of stress-responsive genes and induced salinity tolerance in Arabidopsis	(Jithesh et al., 2019)
6	Acadian Marine Plant Extract Powder (Acadian Seaplants Limited)	*Arabidopsis thaliana* (Col-0)	Improved growth of Arabidopsis in saline and phosphorus deprived conditions.	(Shukla et al., 2018)
7	*A. nodosum* (Biovita) extract (PI industries)	Mung bean (*Vigna radiata* L.)	Enhanced the antioxidant activities in mung beans under salt stress	(Kumari et al., 2021)
8	Rygex (Agriges S.R.L.) and Superfifty (BioAtlantis Ltd.)	Tomato (*Solanum lycopersicum*)	Modulated amino acids and potassium levels and improved salt tolerance in tomato	(Dell'Aversana et al., 2021)
9	Rygex (Agriges S.R.L.) and Superfifty (BioAtlantis Ltd.)	Tomato (*Solanum lycopersicum*)	Increased the accumulation of macro and micro-nutrient contents of tomato under salt stress	(Di Stasio et al., 2018)
10	Acadian®	Passion fruit (*Passiflora edulis* Sim.)	Improved the initial growth of passion fruit under saline stress	(Sá et al., 2018)
11	*A. nodosum* extract	Avocado (*Persea americana* Mill.)	Mitigated salinity stress in avocado plant and increased potassium and calcium concentrations in leaves	(Bonomelli et al., 2018)
12	Acadian Marine Plant Extract Powder (Acadian Seaplants Limited)	Grape (*Vitis vinifera*)	Facilitated grape plant recovery after a drought stress period	(Tombesi et al., 2021)

Table 4. Effects of different A. nodosum extracts on various plants and soil systems.

S. No.	Extract	Crop	Function	Reference
13	Stimplex (Acadian Seaplants Limited)	Tomato Sweet pepper	Decreased disease severity and increased expression of defence-related genes and enzymes	(Ali et al., 2019)
14	*A. nodosum* extract (Acadian Seaplants Limited)	wheat (*Triticum aestivum*)	Reduced disease severity and increased the expression of pathogenesis-related genes and the activity of defence-related enzymes	(Gunupuru et al., 2019)
15	*A. nodosum* extract (Acadian Seaplants Limited)	Pea (*Pisum sativum* L.)	inhibited pathogen development and increased expression of defence-related genes and enzymes	(Patel et al., 2020)
16	Ativa Power® (Alternativa Agrícola)	soybean (*Glycine max* (L.) Merrill)	Reduced the root-knot nematode population density and enhanced the activity of defence-related enzymes	(Rinaldi et al., 2021)
17	Stella Maris® (Acadian Seaplants Limited)	Tomato Pepper	Improved plant growth and increased rhizosphere microbial biodiversity in soil	(Renaut et al., 2019)
18	*A. nodosum* extract (Seasol Plus Calcium)	Tomato (*Lycopersicon esculentum*)	Improved growth and productivity of tomato and increased microbial biomass in soil	(Hussain et al., 2021)
19	*A. nodosum* extract (Acadian Seaplants Limited)	Strawberry (*Fragaria* × *ananassa*)	Regulated the activities of defence-related enzymes and reduced disease severity and incidence	(Bajpai et al., 2019)
20	*A. nodosum* extract (Acadian Seaplants Limited)	lettuce (*Lactuca sativa* L.) spinach (*Spinacia oleracea* L.)	Reduced post-harvest losses of lettuce and spinach during the storage	(Sandepogu et al., 2019)

Table 4. Effects of different A. nodosum extracts on various plants and soil systems.

S. No.	Extract	Crop	Function	Reference
21	Sealicit (Brandon Bioscience)	*Arabidopsis thaliana*	Reduced pod shattering and improved pod firmness	(Łangowski et al., 2019)
22	Commercially available Premium liquid Seaweed	Onion (*Allium cepa* L.)	Improved vegetative growth, yield and nutrient content of onion plant	(Hidangmayum & Sharma, 2017)
23	OSMO® (OSMO® International NV)	Tomato (*Solanum lycopersicum*)	Affected hatching and sensory perception of root-knot nematodes	(Ngala et al., 2016)
24	*A. nodosum* extract	*Arabidopsis thaliana* (Col-0)	Protected Arabidopsis from the green peach aphid biotic pressure and delayed senescence in treated plants	(Weeraddana et al., 2021)
25	Stella MarisTM (Acadian Seaplants)	*Calibrachoa* x *hybrida*	Improved vegetative growth of the plant, increased production of bioactive molecules and enhanced antibacterial and antifungal properties of the plant	(Elansary et al., 2016a)
26	*A. nodosum* extract (Acadian Seaplants Limited)	Alfalfa (*Medicago sativa*)	Stimulated root nodulation by improving activity of symbionts	(Khan et al., 2012)
27	*A. nodosum* extract (Acadian Seaplants Limited)	Carrot (*Daucus carota* L.)	Increased the activities of defence-related enzymes and reduced disease severity in plants	(Jayaraj et al., 2008)
28	Actiwave® (Valagro SpA)	Strawberry (*Fragaria* × *ananassa*)	Improved vegetative growth and yield of strawberry	(Spinelli et al., 2010)
29	*A. nodosum* extract (Acadian Seaplants Limited)	Eggplant (*Solanum melongena* L.)	Alleviated salinity stress and enhanced phenolic antioxidant content of eggplant	(Hegazi et al., 2015)
30	Stella MarisTM (Acadian Seaplants)	Mint (*Mentha* × *piperita* L. "chocolate") Sweet basil (*Ocimum basilicum* L. "purple ruffle"	Increased the essential oil content and composition in plants	(Elansary et al., 2016b)

2.3.1 *A. NODOSUM* EXTRACT AND PLANT GROWTH

A. nodosum contains a variety of unique compounds, such as organic compounds, plant hormones, and different types of polysaccharides, which are usually absent or present in small amounts in terrestrial plants (De Saeger et al., 2020; Khan et al., 2009; Mukherjee & Patel, 2020; Sharma et al., 2014; Shukla et al., 2019; Yakhin et al., 2017).

It has been demonstrated that the application of different extracts of *A. nodosum* can improve the growth and productivity of crops. The pre-harvest foliar application of glycine-betaine or an *A. nodosum* based extract increased antioxidant contents, improved fruit quality, and promoted the fruit ripening process of sweet cherry cv. 'staccato' (Gonçalves et al., 2020). Salvi et al. (2019) reported that the foliar application of an extract of *A. nodosum* improved photosynthesis, stomatal conductance, and secondary metabolites during the last three phenological stages of berry development, thereby enhancing the performance and quality of grapes *(Vitis vinifera* cv. Sangiovese) (Salvi et al., 2019). The application of different commercially available *A. nodosum* extracts on maize (*Zea mays* L.) promoted root morphology traits and improved plant nutrition, which may be caused by their high content of hormones (e.g., auxin and cytokinin) and phenol content (Ertani et al., 2018).

2.3.2 *A. NODOSUM* AND PLANT ABIOTIC STRESS TOLERANCE

In addition to promoting plant growth, *A. nodosum* extract can help to manage the abiotic stresses of plants. Zamani-Babgohari et al. (2019) showed that the alkaline extract of *A. nodosum* improved cell growth, membrane stability, and nuclear integrity and reduced cell death of cold-stressed tobacco (*Nicotiana tabacum* L.) cultivar Bright Yellow-2 (BY-2) suspension cultures. This study also revealed that *A. nodosum* extract protected BY-2

cultures from freezing stress by upregulating key freezing tolerance genes, including galactinol synthase 2, pyrroline 5-carboxylate synthase, and acetyl-CoA carboxylase. Studies revealed that the application of various extracts from *A. nodosum* enhanced salinity stress tolerance in *Arabidopsis thaliana* (Jithesh et al., 2019; Shukla et al., 2018), mung bean (Kumari et al., 2021), tomato (Dell'Aversana et al., 2021; Di Stasio et al., 2018), passion fruit (Sá et al., 2018), and avocado (Bonomelli et al., 2018). Jithesh et al. 2019 indicated that the bioactive compounds present in the ethyl acetate fraction of *A. nodosum* modulated the expression of a plethora of genes associated with stress response (e.g., LEA3, LEA1, and CCA1) in *A. thaliana*. Shukla et al. (2018) revealed that such gene expression was achieved by regulating miRNAs involved in stress tolerance. Tombesi et al. (2021) found that the foliar application of *A. nodosum* powder alleviated drought stress in grapevine by increasing plant stomatal conductance and transpiration.

2.3.3 *A. NODOSUM* AND PLANT BIOTIC STRESS TOLERANCE

Seaweed extract contains various bioactive compounds (e.g., laminarin, alginates, and fucans) that have shown a biocontrol ability effective against various plant pathogens, such as bacteria, viruses, fungi, and nematodes (Khan et al., 2009; Nabti et al., 2017; Sharma et al., 2014; Shukla et al., 2016). Several studies have shown that *A. nodosum* extract can enhance plant resistance to various pathogens (Patel & Mukherjee, 2021; Shukla et al., 2019).

The application of a commercially available *A. nodosum* extract (i.e., Stimplex) showed improvement of plant growth parameters as well as reduction of *Xanthomonas campestris* pv. *vesicatoria* and *Alternaria solani* on greenhouse and field tomato and sweet pepper plants in a tropical environment (Ali et al., 2019). Ali et al. (2019) also indicated that the

extract also induced the activities of defence-related enzymes (e.g., phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, chitinase, and β-1,3-glucanase) and enhanced the transcription of genes involved in auxin indole-3-acetic acid (IAA), gibberellin (GA2ox), and cytokinin (IPT) biosynthesis. The severity of Fusarium head blight disease was significantly reduced in wheat seedlings treated with the combination of *A. nodosum* extract and chitosan as drench applications. The treated wheat seedings also have shown increased expression of pathogenesis-related protein 1 and β-1,3-glucanase genes and enhanced the activity of defence-related enzymes, like phenylalanine ammonia-lyase (PAL), peroxidase (PO), and polyphenol oxidase (PPO) (Gunupuru et al., 2019). Similarly, Patel et al. (2020) found that the combined application of *A. nodosum* extract (0.015%) and chitosan (100 ppm) significantly reduced the severity of pea powdery mildew disease caused by *Erysiphe pisi*. The combined application also resulted in increased activity of plant defence enzymes (PAL and PO) and enhanced production of reactive oxygen species and hydrogen peroxide (H_2O_2) through modulating salicylic acid (SA)- and jasmonic acid (JA)-mediated signaling pathways (Patel et al., 2020). It was reported that an aqueous extract of *A. nodosum* effectively reduced the root-knot nematode *Meloidogyne javanica* population density in soybean and *in vitro* egg hatching. The soil-drenching application of *A. nodosum* extract also increased PO, PPO, PAL, and β-1,3-glucanase activities in uninoculated soybean plants (Rinaldi et al., 2021).

2.3.4 *A. NODOSUM* AND SOIL HEALTH

A further function of *A. nodosum* extract is to promote soil health by increasing minerals available to plants and improving soil aeration and water-holding capacity due to the presence of alginates (Craigie, 2011; Illera-Vives et al., 2015; Sharma et al., 2014; Spinelli

et al., 2010). It has been reported that the soil application of a commercially available *A. nodosum* extract (i.e., Stella Maris) increased the rhizosphere microbial biodiversity in soil samples (Renaut et al., 2019). Similarly, Hussain et al. (2021) reported that the application of a seaweed extract made from *Durvillaea potatorum* and *A. nodosum* increased microbial biomass in the soil.

2.4 PLANT GROWTH-PROMOTING RHIZOBACTERIA

Climate change and the rising population pose a serious threat to global food security, increasing demand for agricultural yields (Meena et al., 2020). The agroecosystems are being damaged by the unwise use of synthetic chemicals to provide food to the burgeoning population. It has been recognized that plant growth-promoting rhizobacteria (PGPR) are able to enhance plant growth and development, enhance plant stress tolerance, and improve soil health (Khatoon et al., 2020).

Plant growth-promoting rhizobacteria, first defined by Kloepper & Schroth (1978), was used to describe some rhizobacteria which colonize plant roots and exert a beneficial effect on plant growth. Several PGPR genera were reported in previous studies, such as *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Enterobacter*, *Erwinia, Flavobacterium*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Thiobacillus,* and others (Basu et al., 2021; Hassan et al., 2019). The ideal PGPRs always have the following characteristics: (i) they are able to colonize plant roots efficiently, (ii) they are able to survive, multiply and compete with other microbes in the rhizosphere, and (iii) they are able to enhance plant growth (Ahemad & Kibret, 2014), and (iv) they are safe for the environment and able to tolerate physicochemical factors (Nakkeeran et al., 2006; Vejan et al., 2016).

PGPRs promote plant growth and development through different (direct and indirect) mechanisms, including nitrogen fixation, phytohormones production, and soil minerals (like P, K, Zn, and Fe) solubilization. PGPRs protect plants from abiotic stresses by regulating stress markers and producing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. PGPRs protect plants from biotic stresses through the induction of disease resistance and the production of siderophores, volatile organic compounds, antibiotics, and lytic enzymes (Basu et al., 2021; Goswami et al., 2016; Vejan et al., 2016). Induced resistance has two main forms: systemic acquired resistance (SAR) and induced systemic resistance (ISR). In both forms, plant defences are preconditioned by prior infection or treatment, which contributes to the development of resistance against subsequent exposure to a pathogen or parasite (Meena et al., 2020). Systemic acquired resistance pathway depends on SA and Pathogenesis-related (PR) proteins. PGPRs can elicit ISR in plants, which is regulated by jasmonate and ethylene and commonly without the involvement of PR proteins (Meena et al., 2020; Vlot et al., 2021). PGPR-mediated ISR protects plants against challenging pathogens by modifying cell wall structure, changing the physiological and biochemical reaction of the host plant, and synthesizing defence chemicals (Ramamoorthy et al., 2001). Several published reports have shown the biocontrol effect of different PGPRs in many crop diseases. Ghadamgahi et al. (2022) found that *Pseudomonas aeruginosa* FG106 showed antagonism against the tested plant pathogens (*Alternaria alternata*, *Botrytis cinerea*, *Clavibacter michiganensis* subsp. *michiganensis*, *Phytophthora colocasiae*, *Phytophthora infestans*, *Rhizoctonia solani*, and *Xanthomonas euvesicatoria* pv. *perforans*). It has also been found that *P. aeruginosa* (strain FG106) is capable of producing proteases and lipases, as well as inducing high

phosphate solubilization, producing siderophores, ammonia, IAA, and HCN, and forming biofilms that enhance plant growth and biocontrol activity. It has been reported that the crude 1-butanol extract of *B. subtilis* 30VD-1's cell-free culture filtrate significantly reduced the radial growth of *Fusarium* spp. under *in vitro* conditions, reduced Fusarium wilt severity in pea plants, and increased plant dry mass. *B. subtilis* 30VD-1 was also observed to produce chitinase, volatiles, and other antifungal compounds, suggesting that it has a multivariate biocontrol mechanism against phytopathogen (Khan et al., 2018). Both *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34 applications caused a reduction in the disease severity of potato wilt and an increase in crop yield. The induction of defence-related enzymes and the accumulation of total phenol and SA were also noticed in biocontrol agents treated in potato plants, indicating the induction of systemic resistance. In addition, both *E. cloacae* PS14 and *T. asperellum* T34 were proven to be able to produce siderophore, IAA, hydrogen cyanide, and SA under *in vitro* conditions (Mohamed et al., 2020).

In addition to promoting plant growth and development, PGPRs also play another important role in environmental cleanup (soil bioremediation). PGPRs can degrade heavy metals (like Cd, Hg, and Zn) through their intrinsic properties or convert heavy metals from toxic to non-toxic forms (Gupta & Joia, 2016; Kabiraj et al., 2020; Ledin, 2000). Table 5 shows some examples of the effects of PGPRs on plants. PGPR strains such as *Bacillus* spp. and *Pseudomonas* spp., which have been shown to be effective biocontrol agents, have been commercialized (Haas & Défago, 2005). In Canada, some PGPR-based formulations are currently available commercially (Table 6), including Nodulator® LQ (*Bradyrhizobium japonicum*) for increasing yield potential in soybean; Serenade® SOIL

(QST 713 strain of *B. subtilis*) for the suppression of soil diseases caused by *Fusarium* spp., *Pythium* spp., *Phytophthora* spp., and *Rhizoctonia* spp.; and Soil ActivatorTM (*B. subtilis, Bacillus amyloliquefaciens*, *Pseudomonas monteilii*) for increasing crop yield (BASF, 2022; Bayer Crop Science, 2021b; Earth Alive Clean Technologies, 2022).

Table 5. List of the effect of plant growth-promoting rhizobacteria application on different plants.

	PGPR	PGPR Mechanisms	Plants	Findings	References
1	*Azospirillum brasilense*, *Burkholderia ambifaria, Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae*	Nitrogen fixation IAA synthesis	Onion (*Allium cepa* L.)	The bacterial seed inoculation increased vegetative growth, total phenolic contents, and antioxidant activities of onion plants	(Pellegrini et al., 2021)
2	*Bacillus megaterium*	Phosphate solubilization IAA synthesis Nitrogen fixation	Tomato (*Lycopersicon esculentum*)	Bacterial soil application enhanced shoot length in tomato plants in a dose-dependent manner	(Yavarian et al., 2021)
3	*Enterobacter* sp. 15S, *Pseudomonas* sp. 16S	IAA synthesis	Tomato	Root and shoot dry biomass in tomato plants were significantly increased by both bacterial inoculations	(Zuluaga et al., 2021)
4	*Bacillus subtilis* BERA 71	Induction of osmoprotectants production	Chickpea (*Cicer arietinum* L.)	Bacterial inoculation suppressed ROS generation and lipid peroxidation and improved proline accumulation in treated plants.	(Abd_Allah et al., 2018)

Table 5. List of the effect of plant growth-promoting rhizobacteria application on different plants.

	PGPR	PGPR Mechanisms	Plants	Findings	References
5	*Bacillus subtilis*, *Bacillus siamensis*, *Brevibacillus gelatini*, *Pseudomonas geniculata*, *Pseudomonas beteli, Burkholderia ubonensis, Burkholderia territorii*	Lytic enzymes production Phosphate solubilization ACC deaminase production Ammonia production	Black pepper (*Piper nigrum* L.)	Improved plant growth and nutrient uptake states in black pepper plants	(Lau et al., 2020)
6	*Pseudomonas reactans* EDP28, *Pantoea alli* ZS 3-6	Phytohormones and organic acids production Siderophore production Ammonia production	Maize	Improved growth and nutrient status in maize plants under salinity stress	(Moreira et al., 2020)
7	*Bacillus cereus* SA1	Phytohormones and organic acids production	Soybean	The biomass, chlorophyll content and chlorophyll fluorescence of soybean plants were improved by bacterial inoculation under normal and heat stress conditions	(Khan et al., 2020)
8	*Bacillus subtilis* SBMP4, *Lysinibacillus fusiformis* NBRC15717, *Achromobacter xylosoxidans* NBRC15126	The induction of the systemic resistance Siderophores production HCN production	Tomato	PGPR isolates improved the morphological and yield characters and reduced disease severity in infected tomato plants	(Attia et al., 2020)

Table 5. List of the effect of plant growth-promoting rhizobacteria application on different plants.

	PGPR	PGPR Mechanisms	Plants	Findings	References
9	*Bacillus subtilis Rhizo* SF 48	ACC deaminase production	Tomato (*Solanum lycopersicum* L.)	Alleviated the oxidative damages of tomato plants and improved plant groath under drought stress	(Huang et al., 2019)
10	*Enterobacter cloacae* CTWI-06	Nitrogen fixation P, K, and Zn solubilization Ammonification IAA synthesis Antifungal activities	Rice (*Oryza sativa*)	Enhanced growth traits and productivity of rice	(Pattnaik et al., 2020)
11	*Enterobacter cloacae* MG00145	IAA synthesis Phosphate solubilization Siderophore production Ammonia production	Rice (Oryza sativa) Groundnut (*Arachis hypogae*a) Black gram (*Vigna mungo*) Totia (*Brassica rapa*)	Improved seed germination rate, shoot and root biomass, and seed vigour index in four selected crops	(Panigrahi et al., 2020)
12	*Bacillus endophyticus* (KT379993), *Bacillus cereus* (KT379994)	Lytic enzymes production Antibiotics production	Turmeric (*Curcuma longa*)	Reduced the disease incidence of rhizome rot and increased plant growth and rhizome biomass	(Chauhan et al., 2016)
13	*Bacillus sonorensis*	Phosphate solubilization IAA synthesis s Siderophore production Lytic enzymes production HCN production	Chilly (*Capsicum annuum* L.)	The growth, nutrition and fruit yield of chilly plants were significantly improved by bacterial inoculation	(Thilagar et al., 2018)

Table 5. List of the effect of plant growth-promoting rhizobacteria application on different plants.

	PGPR	PGPR Mechanisms	Plants	Findings	References
14	*Aneuriniba cillus aneurinilyti cus*	ACC deaminase production	French Bean (*Phaseolus vulgaris*)	Promoted the growth of French bean seedlings under salinity stress conditions	(Gupta & Pandey, 2019)
15	*Pseudomon as putida*	ACC deaminase production	Poppy (*Papaver somniferum* L.)	Reduced disease severity and improved the growth and yield of poppy plants	(Barnawal et al., 2017)
16	*Stenotroph omonas maltophilia*	ACC deaminase production Gibberellic acid production IAA synthesis Siderophore production Phosphate solubilizatio n The induction of the systemic resistance	Wheat (*Triticum aestivum*)	Bacterial inoculation improved wheat plant growth and increased defence-related enzymes activities	(Singh & Jha, 2017)
17	*Pseudomon as fluorescens* CHA0	The induction of the systemic resistance	Tomato (*Solanum lycopersicum* L.)	Improved growth parameters of tomato plants and reduced the infection of root-knot nematodes in tomato plants	(Dehghanian et al., 2020)
18	*Pseudomon as protegens* CHA0-ΔretS-nif	Nitrogen fixation	Garlic (*Allium sativum* L.)	Bacterial inoculation significantly promoted growth and yields of garlic	(Wang et al., 2020)

Table 5. List of the effect of plant growth-promoting rhizobacteria application on different plants.

	PGPR	PGPR Mechanisms	Plants	Findings	References
19	*Serendipita Indica, Pseudomonas Protegens* CHA0-mCherry	The induction of the systemic resistance	Wheat (*Triticum aestivum* L.)	Bacterial inoculations promoted root growth and stem biomass and reduced the spore density in infected plants	(Ashrafi et al., 2021)
20	*Pseudomonas frederiksbergensis* OS261	Induction of antioxidant capacity	Tomato (*Solanum lycopersicum* L.)	Reduced membrane damage and activated antioxidant enzymes and proline synthesis in the tomato leaves under chilling temperature conditions	(Subramanian et al., 2016)
21	*Bacillus subtilis*	Peptide antibiotics production	Potato (*Solanum tuberosum*)	The bacterial applications effectively reduced disease incidence of late blight of potato as well as increased plant vegetative parameters of potato under field condition	(Kumbar et al., 2019)
22	*Enterobacter cloacae* PS14	Antibiotics production IAA synthesis Siderophore production HCN production Salicylic acid production The induction of the systemic resistance	Potato (*Solanum tuberosum*)	Inhibited the growth of *Ralstonia solanacearum* (Smith) *in vitro*, reduced the disease severity of potato bacterial wilt, improved crop yield under greenhouse and field conditions as well as increased defence-related enzyme activities, the total phenol and salicylic acid contents in potato plants	(Mohamed et al., 2020)

Table 5. List of the effect of plant growth-promoting rhizobacteria application on different plants.

	PGPR	PGPR Mechanisms	Plants	Findings	References
23	*Bacillus subtilis 30VD-1*	Lytic enzymes production Diffusible and volatile antifungal compounds production	Pea (*Pisum sativum*)	Inhibited the growth of *Fusarium* spp. *in vitro*, reduced wilt severity in pea plants, and increased dry plant biomass under *in vivo* condition	(Khan et al., 2018)
24	*Pseudomonas aeruginosa* FG106	Lytic enzymes production Phosphate solubilization Siderophore production Ammonia production IAA synthesis HCN production	Tomato (*Lycopersicon esculentum*, Mobil)	Bacterial root dipping treatment reduced disease index of tomato plants caused by *Rhizoctonia* solani, *Clavibacter michiganensis* subsp. *Michiganensis and Xanthomonas euvesicatoria pv. perforans* exposures and increased fresh and dry weights for root and shoot of tomato plants under greenhouse condition	(Ghadamgahi et al., 2022)

Table 6. List of commercial PGPR products.

Products	PGPR	Beneficial effects	References
NTS Bio-P™	*Azotobacter* species and *Bacillus subtilis*	Improves phosphorus availability, root growth and strength, increases Brix levels, quality, and yield of crops.	(AGRICULTURE SOLUTIONS INC., 2022)
SYNTHOS®	Bacillus spp.	Improves phosphorus availability and promotes plant growth	(Koch Agronomic Services, 2022)

Table 6. List of commercial PGPR products.

Products	PGPR	Beneficial effects	References
Soil Activator™	*Bacillus subtilis, Bacillus amyloliquefaciens, Pseudomonas monteilii*	*Improvse nutrient availability and uptake, and soil moisture retention*	(Earth Alive Clean Technologies, 2022)
BTK	*Bacillus thuringiensis* (var. kurstaki)	Control all types of caterpillar pests	(Canada, 2009)
Stargus	*Bacillus amyloliquefaciens* strain F727	Control certain foliar (e.g., downy mildew and botrytis) and soil-borne diseases (e.g., Fusarium)	(Marrone Bio Innovations, 2022)
Actinovate® SP	*Streptomyces lydicus* strain WYEC 108	Provides control of soil-borne fungi (e.g., Fusarium, Rhizoctonia and Pythium) and foliar disease (e.g., powdery mildew, botrytis and sclerotinia)	(Novozymes, 2022)
Utrisha™ N	*Methylobacterium symbioticum* SB23	Improves nitrogen efficiency	(Corteva Agriscience, 2022)
Serenade OPTI	*Bacillus subtilis* (strain QST 713)	Control many fungal (e.g., Botrytis, Powdery mildew and Early blight) and bacterial disease (e.g., Bacterial blight and Bacterial spot)	(Bayer Crop Science, 2021a)
Serenade SOIL	*Bacillus subtilis* (strain QST 713)	Protects against soil diseases such as fusarium, rhizoctonia and pythium	(Bayer Crop Science, 2021b)
CEASE	*Bacillus subtilis* (strain QST 713)	Control common fungal (e.g., Botrytis, powdery mildews and downy mildews) and bacterial diseases (e.g., Pseudomonas, and Xanthomonas)	(BioWorks, 2022)

PGPRs have great potential to become the safest solution to increase agricultural productivity due to their positive effects on biofertilization, biocontrol, and bioremediation (Vejan et al., 2016). However, to truly succeed in the field, the development of PGPR formulations still needs to overcome many challenges and limitations, such as the optimization of bacterial isolates, biosafety of PGPRs, and biological activity under field applications (Basu et al., 2021; Meena et al., 2020).

CHAPTER 3 : MATERIALS AND METHOD

3.1 PLANT MATERIALS

Onion seeds (Utah Sweet Spanish), onion sets (Yellow Dutch), and Pro-mix BX (without mycorrhizae) were purchased from Halifax Seed Company (Halifax, NS, Canada). The onion seeds and sets were surface sterilized by using 1% sodium hypochlorite (NaOCl) for 3 min., washed three times with sterilized distilled water (SDW), and then imbibed in SDW for stratification. The seeds and sets were planted in Pro-mix in a growth chamber that adjusted 22±2°C (day/night) with a photoperiod of 16 h (cool white fluorescent tube lights, 150 µmol m^{-2} s^{-1}). The growth chamber was located in the Department of Plant, Food, and Environmental Sciences at Dalhousie Agricultural Campus (Truro, NS, Canada).

3.2 ISOLATION AND IDENTIFICATION OF *FUSARIUM OXYSPORUM* ISOLATES

Isolates of FOC were obtained from naturally infected onion plants collected at Sawler Farms (Berwick, NS, Canada). Onion tissues taken from bulb scales were surface sterilized by dipping in 70% ethanol for 3 min, then submersed in 1% NaOCl for 3 min, followed by rinsing with SDW three times. The sterilized tissues were surface dried with sterilized filter paper and then cultured on potato dextrose agar medium (PDA; 4 g potato infusion, 20 g dextrose, and 15 g agar in 1 L distilled water, pH 5.6) and incubated at 28±2°C for 3-5 days. Fungal mycelia grown from tissues that are similar to known FOC were subcultured on new PDA plates. The fungal cultures were first identified and characterized based on the characteristics of three types of spores (microconidia, macroconidia, and chlamydospores) and colony growth (Marasas et al., 1984).

The pure cultures of each FOC isolate (7- to 10-day-old) were used for DNA extraction. Mycelia were scraped from the surface of the pure cultures and ground in liquid nitrogen. The fungal DNA of each isolate was extracted using the GeneJet Plant Genomic DNA Purification Mini Kit (Thermo Scientific, USA) as per the manufacturer's protocol. DNA concentration and purity were measured using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA). The internal transcribed spacer (ITS) region was amplified using primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′). The polymerase chain reaction (PCR) was performed in a total 50 µl reaction mixture containing 25 µl EconoTaq® Plus Green 2X Master Mix (Lucigen, USA), 2.5 µl of 10µM forward primer (ITS1), 2.5 µl of 10µM reverse primer (ITS4), 3 µl of template DNA (50 ng/µl), and 17 µl DNase/RNase-Free distilled water. The PCR cycling conditions were set as initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 20 s, extension at 72°C for 30 s, and final extension at 72°C for 7 min. Then, the PCR amplified products were subjected to electrophoresis on a 0.8% agarose gel stained with Gel red Nucleic Acid Gel Stain (Thermo Scientific, USA). GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA) was loaded as a size standard to determine the size of PCR products. Then, the PCR amplified products were purified with a PCR purification kit and sequenced at the Centre for Applied Genomics (TCAG) SickKids Hospital, Toronto. The obtained sequence data were subjected to NCBI web BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their identity.

Living strains of isolated FOC were submitted to the Canadian Collection of Fungal Cultures (DAOMC, Ottawa, Canada).

3.3 MAINTENANCE OF PATHOGENIC FUNGI AND PGPR AND PREPARATION OF ANE SOLUTIONS

The isolates of FOC (pathogenic fungi) were cultured and maintained on PDA media at 28°C. The inoculum/spore suspension of each isolate was prepared from fresh, mature (5- to 10-day-old) cultures of each isolate and SDW. SDW was added to fungal culture plates, and fungal spores were scraped off the culture with an L-shape spreader. The spore suspension was filtered through sterile cotton wool, then quantified with the aid of a hemocytometer, and diluted with SDW to maintain the concentration of $1x10^5$ spores/ml.

The PGPRs used in the present study i.e., *Pseudomonas protegens* CHA0 (CHA0) strain and *Enterobacter cloacae* CAL2 (CAL2) were obtained from Dr. Zhenyu Cheng, Department of Microbiology Immunology, Faculty of Medicine, Dalhousie University. *Bacillus subtilis* (BS) was previously isolated from soil. *Pseudomonas protegens* CHA0 was cultured and maintained on King's B agar (KMA; 10 g agar, 20 g mixed peptone, 1.5 g anhydrous K_2HPO_4, 1.5 g $MgSO_4$, 10 ml glycerol in 1 L distilled water, pH 7.2) at 28±2°C. *Enterobacter cloacae* CAL2 was cultured and maintained on Trypticase soy agar (TSA; 17 g Tryptone, 3 g soytone, 2.5 g glucose, 5 g NaCl, 2.5 g K_2HPO_4, 15 g agar in 1 L distilled water, pH7.3) at 28±2°C. *Bacillus subtilis* was cultured and maintained on Nutrient agar (NA; 5 g pancreatic digest of gelatin, 3 g beef extract, and 15 g agar in 1 L distilled water, pH7.0) at 28±2°C

ANE powder was provided by Acadian Seaplants Limited (Dartmouth, NS, Canada). A stock solution of ANE (1% and 0.5%) was prepared by dissolving 1g and 0.5 g of ANE powder in 100 ml of SDW, respectively, and stored at 4°C until further use. Different concentrations of ANE solution were prepared by using the stock solution.

3.4 PATHOGENICITY TESTS

3.4.1 ONION BULB PATHOGENICITY TEST

Yellow Dutch onion bulbs purchased from a local store were used to test the pathogenicity of the two FOC isolates according to the protocol described (Kalman et al., 2020). Onion bulbs were sterilized in 70% ethanol and dried in a laminar flow hood. Spore suspension (0.2 ml, 10^5 spores/ml) of each FOC isolate was prepared and injected two times into two different areas of the basal region of each surface-sterilized onion bulb by using a plastic syringe (1 ml) with a needle (1.5 inches). Control bulbs were injected with a similar volume of SDW. Each bulb was placed individually in a closed sterilized plastic bag to maintain moisture and prevent contamination and then incubated at 28±2°C in the dark. Disease symptoms were photographed after one week of incubation, and the fungus from each infected onion was isolated on PDA and identified.

3.4.2 ONION SEEDLING PATHOGENICITY TEST

The pathogenicity tests of each FOC isolate were also performed on healthy onion seedlings. The onion (Utah Sweet Spanish) seeds were sterilized by soaking in 1% NaOCl for 3 min and then rinsed three times in SDW. Surface-sterilized onion seeds were transferred to a sterile Whatman paper in a Petri dish (Each Petri dish with 15 seeds). The seeds were inoculated with 6 ml spore suspension (10^5 spores/ml) of each FOC isolate. SDW (6 ml) was added to the control group. The plates were incubated at 28±2°C in the dark for 9 days, and SDW was added to each plate every three days to maintain moisture and to allow efficient germination and disease progression. The seeds were photographed, and the germination percentage was measured. The experiment was repeated three times with four replicates.

Another two different inoculation methods (see Appendix A) were used to test the pathogenicity of two isolates to onion seedlings.

3.4.3 POT PATHOGENICITY TEST

Different methods were used to evaluate the pathogenicity test of FOC isolate in onion plants *in vivo* (see Appendix B) and to obtain symptoms in the plants.

Onion sets (Yellow Dutch) were sterilized by soaking in 1% NaOCl for 3 min and then rinsed three times in distilled water. Then, onion sets were placed on wet sterile cheesecloth in plant growing trays which were placed in a growth chamber adjusted to 22±2°C (day/night) with a photoperiod of 16 h (cool white fluorescent tube lights, 150 µmol m^{-2} s^{-1}). The growth chamber was located in the Department of Plant, Food, and Environmental Sciences at Dalhousie Agricultural Campus (Truro, NS, Canada). After one week of germination, the germinated onion sets were inoculated with spore suspensions (10^5 spores/ml) of each FOC isolate by root dipping method for 30 min. For the control group, onion roots were dipped in SDW. Then, onion plants were transplanted into pots filled with Pro-mix at a rate of three plants per pot. The biomass data were collected one month after inoculation, and data were represented as gram FW/plant. All the root tissues were frozen with liquid nitrogen and ground with pestle and mortar, and then stored in deep freezer (-80°C) for further analysis. The experiment was repeated twice with 6 replicates.

The relative amount of pathogen in onion roots was quantified as previously described (Gachon & Saindrenan, 2004). Approximately 100 mg of liquid nitrogen-ground onion root sample was used for DNA extraction. DNA was extracted from each sample and two FOC isolates (Fa and Fb) according to the manufacturer protocol using “Gene JET

Genomic DNA Purification Kit" (Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA concentration and purity were measured using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA).

The primer sets used to quantify FOC DNA were OMP1049 (5′-TGCGATTTGGACGAG ATATGT-3′) and OMP1050 (5′-CTCATTTGCCTACCCTGTACCTACC-3′) (Wang et al., 2019), and the primer sets used to quantify onion DNA were Ac Actin R (5′-CTGGGATGACATGGAGAAGATT-3′) and Ac Actin F (5′-GTTAAGTGGAGCC TCCGT-3′) (Khosa et al., 2016).

Real-time PCR was performed in a StepOne Plus Real-Time PCR system (Applied Biosystems, Mississauga, ON, Canada). The PCR reactions were performed in 96-well reaction plates (Applied Biosystems, Mississauga, ON, Canada). Each reaction (10 µl) contained iTaq 2X SYBR Green mix (Bio-Rad, Mississauga, ON, Canada) 5 µl, 0.25µM of each primer, 3 µl of DNA (10 ng/ul), and 1.5 µl of DNase/RNase-Free Distilled Water. In negative controls, the DNA was replaced by the same amount of DNase/RNase-Free Distilled Water. The PCR cycling conditions were 95°C for 10 min, followed by 40 cycles of amplification (95°C for 15 s and 59°C for 1 min). Then, the melt curve stage was set at 95°C for 15 s, 59°C for 1 min and 95°C for 15 s.

The standard curve for FOC and onion DNA was developed using a triplicate test of a series of known diluted concentrations (2000, 200, 20, 2, and 0.2pg/µl) of each FOC isolates (Fa and Fb) and onion DNA, respectively. The quantification of FOC and onion was calculated from cycle threshold (Ct) values using the standard curves. The relative amount of fungal DNA was normalized by calculating the ratio between fungal DNA and onion DNA.

According to the results of pathogenicity tests and sequencing analysis, one of the FOC isolates was selected for *in vitro* and greenhouse experiments.

3.5 EFFECT OF ANE ON THE GROWTH OF THREE PGPR STRAINS

The effect of ANE on the growth of three PGPR strains was investigated according to the spectrophotometric assay described previously (Raghupathi et al., 2011). The bacterial growth curve was obtained by measuring changes in the optical density (OD) of bacterial culture over time. OD measurements were made using a Cytation^{TM} 5 (BioTek, Winooski, VT) cell imaging multimode plate reader at 600 nm. The stock culture of each PGPR strain (OD600=0.6) was diluted to 1:100 with fresh broth, which was supplemented with different concentrations of ANE (0%-Control, 0.001%, 0.01%, and 0.1%). Liquid broth supplemented with different concentrations of ANE (0%, 0.001%, 0.01%, and 0.1%) without any bacteria inoculated were served as blank. The diluted cell mixtures and blank solutions were subsequently plated as needed into a 96-well microplate with a total volume of 300 µl. The 96-well microplate was incubated in the Biotek Cytation^{TM} 5 multimode plate reader at 26 ˚C with linear shaking at 567 cpm (3 mm) for 24 h. The OD of the bacterial cultures was taken at 600 nm every 30 min, and the background (turbidity due to LB broth and ANE) was eliminated by taking blank readings. The experiment was replicated three times with 3 replicates.

3.6 EFFECT OF PGPR AND/OR ANE ON MYCELIAL GROWTH OF FOC

Different inoculation methods and culture media (see Appendix C) were used to select the appropriate dual culture method to enable the development of pathogen and PGPR strains (biocontrol agents) which better reflect the antagonistic activity of PGPR strains. We observed that the PGPR strain grew very slowly using the agar plug inoculation method

compared to the streaking inoculation method, which may affect the antagonistic activity of the PGPR strain. We also observed that there was no pigmentation of FOC in bacteria media (Table C5-6 and Figure C4-5), which indicated that bacteria media might affect the secondary metabolites production of FOC. Therefore, we used the streaking inoculation method and PDA medium for the dual culture assay in the following experiments.

PGPR and ANE were tested against FOC by using dual culture bioassays with some modifications (Dennis & Webster, 1971). Each PGPR was cultured into the liquid broth and incubated overnight at 28±2˚C, 180 rpm. The overnight culture of each PGPR strain was diluted to 1:100 with fresh broth and incubated at 28±2˚C, 180 rpm till the OD_{600} reached 0.6, which was used as stock culture. The stock culture of each PGPR strain ($OD_{600=0.6}$) was diluted to 1:100 with fresh broth, which was supplemented with different concentrations of ANE (0% and 0.1%). After 10 h incubation (28±2˚C, 180 rpm), 10 µl of each bacterial culture was streaked in a straight line (5 cm) 2 cm away from the edge of a new PDA plate one day before the FOC inoculation. An agar plug (5 mm) of FOC was cut from the growing edge of a seven-day-old culture of FOC and inoculated 4 cm away from the bacterial inoculation. Plates inoculated with fungal discs and clean liquid broth were used as control. The experiment was repeated three times with 3 replicates. The plates were placed into the incubator (28±2°C) for 7/10 days, and then the inhibition zone was measured. The inhibition percentage was calculated by using the equation (Muniroh et al., 2019):

$$\text{Inhibition percentage (\%)} = \frac{R_c - R_t}{R_c} \times 100\%$$

Where:R_c was the radial growth of the fungal colony in the control plate and R_t was the radial growth of the fungal colony in the presence of PGPR and/or ANE

3.7 EFFECT OF PGPR AND/OR ANE ON SPORE GERMINATION OF FOC

The spore germination test was accomplished by using the hanging drop method with some modifications (Fang, 1998). Prior to growth experiments, the stock culture of each PGPR strain ($OD_{600=0.6}$) was diluted to 1:100 with fresh broth and incubated for 10 h incubation (28±2˚C, 180 rpm). The bacterial cultures were centrifuged into pellet bacteria cells. The bacterial cells were washed three times by resuspending the cell pellet in Phosphate-buffered saline (PBS; pH=7.2) and centrifuging. Then, the bacterial suspension was prepared by resuspending the cells in SDW to OD_{600}=0.1 at a density of 10^5~10^6 c.f.u./ml. Pathogen spore suspension (40 µl, 10^5 spores/ml) with 40 µl of each treatment (Bacterial suspension 10^5~10^6 c.f.u./ml, 0.1% ANE, 0.1% ANE combined with bacterial suspension) were kept in pits of the slide. The slides were kept in a moist chamber and incubated for 6 h at 28±2°C. Spore morphology was observed in five different views under a compound light microscope. A total of 100 spores were randomly counted and recorded in each view. The number of germinated spores among those spores in each view was counted and recorded. Germination was considered when the length of the germ tube reached half the size of the spore. The experiment was repeated three times with 3 replicates. The germination rate was calculated as follow:

$$\text{Germination rate (\%)} = \frac{\text{Total counted germinated spores in five views}}{\text{Total counted spores in five views}} \times 100\%$$

Another two different methods (see Appendix D) were tried to assess the effect of PGPR and/or ANE on spore germination of FOC. We observed that bacterial broth (LB and KB broth) had an inhibitory effect on fungal spore germination. Meanwhile, we were not able to accurately quantify living bacterial cells in bacterial suspension if we scrubbed bacteria

directly from bacterial cultures. Therefore, we decided to wash bacterial cells grown in broth cultures when they were at the mid-log growth phase.

In addition to the above *in vitro* experiments, the evaluation of the activity of antifungal substances produced by *P. protegens* CHA0 in the mycelial growth of FOC (Appendix E) was attempted. However, the cell-free supernatant from *P. protegens* CHA0, which was grown in LB broth or LB broth amended with 0.1% ANE, did not show any significant antifungal activity against the mycelial growth of FOC.

3.8 EFFECT OF *E. CLOACAE* AND/OR ANE ON ONION FBR DISEASE UNDER GREENHOUSE CONDITION

In the greenhouse study, one of the PGPR strains most effective against FOC under *in vitro* tests was used. As mentioned in Part 3.4.3, one of the FOC isolates was selected for *in vitro* and greenhouse experiments based on the results of pathogenicity tests and sequencing analysis.

A preliminary test was conducted to evaluate the effect of seed treatment with *E. cloacae* and/or ANE formulations on onion FBR disease under *in vivo* conditions (Appendix F). However, since almost all pathogen-inoculated seeds did not germinate, no plant tissue was available for subsequent biochemical and pathogen quantification analyses. Therefore, onion seedlings were chosen for the greenhouse experiment.

3.8.1 PREPARATION OF INOCULUMS

Inoculum suspension was prepared as described in Part 3.3.

3.8.2 PREPARATION OF TREATMENTS

Enterobacter cloacae CAL2 strain was cultured on Tryptic Soy agar medium and incubated for 3-5 days at 28±2°C. Bacterial colonies were harvested by flooding plates with SDW and gently scraped off the colonies with an inoculation loop. *Enterobacter cloacae* CAL2 stock suspension contained 10^8 c.f.u./ml. The 1% stock solution of ANE was diluted with SDW to prepare ANE treatments and the combined treatment of ANE and *E. cloacae* CAL2. Each treatment included with and without pathogen inoculation groups. The experimental treatments are listed in Table 7.

Table 7. *E. cloacae* CAL2 and ANE alone or their combination are evaluated for their efficiency.

	Treatments	Concentration of ANE (%)	Concentration of *E. cloacae* CAL2(c.f.u./ml)	Other
With pathogen inoculation	T1(Control)	-	-	Water
	T2(0.5% ANE)	0.5	-	-
	T3 (*E. cloacae* CAL2)	-	10^8	-
	T4 (*E. cloacae* CAL2+0.5% ANE)	0.5	10^8	-
Without pathogen inoculation	T1(Control)	-	-	Water
	T2(0.5% ANE)	0.5	-	-
	T3 (*E. cloacae* CAL2)	-	10^8	-
	T4 (*E. cloacae* CAL2+0.5% ANE)	0.5	10^8	-

3.8.3 EXPERIMENTAL DESIGN

The experimental design was a completely randomized design (CRD) that was replicated twice with 12 replications. The experimental units were 384 onion seedlings with four treatment combinations meaning 4 seedlings per replicate and 48 seedlings per treatment.

3.8.4 PLANT TREATMENT AND INOCULATION

Onion plants (two-month-old) were root-dipped into each treatment for 4 hours, and then the treated plant roots were wrapped in cheesecloth and incubated at room temperature (23±2°C) overnight. On the next day, for the group with the pathogen inoculation, inoculum suspension (FOC suspension) was mixed into Pro-mix (10^5spores/g of Pro-mix) and then distributed into each pot. For the group without pathogen inoculation, the same amount of SDW as the inoculum suspension was added to Pro-mix and then distributed into each pot. Then, treated onion plants were transplanted into pots at a rate of four plants per pot.

3.8.5 DATA AND SAMPLE COLLECTION

For enzyme and biochemical assays, root samples were collected at different time points (24 h, 48 h, and 72 h) after the pathogen inoculation. For the root pathogen quantification assay, root samples were collected one month after the pathogen inoculation. Onion plants were carefully removed from the pot. The roots were washed and collected. All the root samples were frozen rapidly by submerging them in liquid nitrogen and ground with pestle and mortar, and then stored in deep freezer (-80°C) for further assays. Biomass data (Fresh weight of shoot and root) were also measured and recorded one month after the pathogen inoculation.

3.8.6 BIOCHEMICAL ANALYSIS

The chemical components of ANE and metabolites produced by PGPR are known as elicitors for induced resistance in plants. The defence-related enzymes and biochemicals were analyzed to evaluate if the ANE and PGPR induce plant defence response in onions.

The experiment included four biological replicates and three technical replicates for individual biological replicates.

Peroxidase activity assay

The peroxidase (PO) activity assay was performed following the previously published protocol (Hammerschmidt et al., 1982). The liquid nitrogen-ground onion root sample (1g) was extracted in 0.1M sodium phosphate buffer (2 ml, pH 7.0, 4°C). The sample was centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, the supernatant was collected and used as the enzyme extract. The reaction mixture included 0.1M sodium phosphate buffer (2.8 ml, pH 7.0), 0.018 M guaiacol (0.05 ml), and enzyme extract (0.1 ml). To initiate the reaction, 1% H_2O_2 (100 µl) was added. The absorbance of the reaction mixture was read at 420 nm by using a Cytation 5 Cell Imaging Multi-Mode Reader (Cytation 5, BioTek, USA) for 3 min at every 30-second interval. The PO activity was calculated according to the following formula (Bo et al., 2017):

$$PO\ activity = \frac{\Delta A_{420} \times V_T}{W \times V_S \times t};$$

ΔA_{420} is the changes of absorbance at 420 nm, V_T is the total volume of the extracted enzyme solution, W is the weight of samples for extraction, V_S is the volume of extracted enzyme solution in the reaction, t is the reaction time. The PO activity was expressed as Unit/min/g of fresh weight (FW) of root tissue.

Phenylalanine ammonia lyases activity assay

Phenylalanine ammonia lyases (PAL) activity was measured using the protocol described by Havir (1987). The liquid nitrogen-ground onion root sample (0.5 g) was extracted in 4 ml borate buffer (0.2 M, pH 8.7, 4°C) and centrifuged at 12,000 rpm for 15 min at 4°C. The

supernatant was used as the enzyme extract. The reaction mixture included 0.5 ml borate buffer, 0.2 ml enzyme extract, 1.3 ml distilled water, and 1 ml 0.1M Phenylalanine. After incubation for 30-60 min at 32°C, 1 M trichloroacetic acid (0.5 ml) was added to stop the reaction. The blank was all reaction mixture except the enzyme extract. The PAL activity was measured at 290 nm by using a UV-Visible spectrophotometer (Pharmaspec UV-1700, Shimadzu, Japan). The amount of trans-cinnamic acid produced was calculated using the standard curve of trans-cinnamic acid and expressed in µg of trans-cinnamic acid per g FW of root tissue. The standard curve of trans-cinnamic acid was generated from known concentrations (0, 1, 2, 4, 5, 6, 8, 9, and 10µg/ml) of trans-cinnamic acid solution (Figure 5).

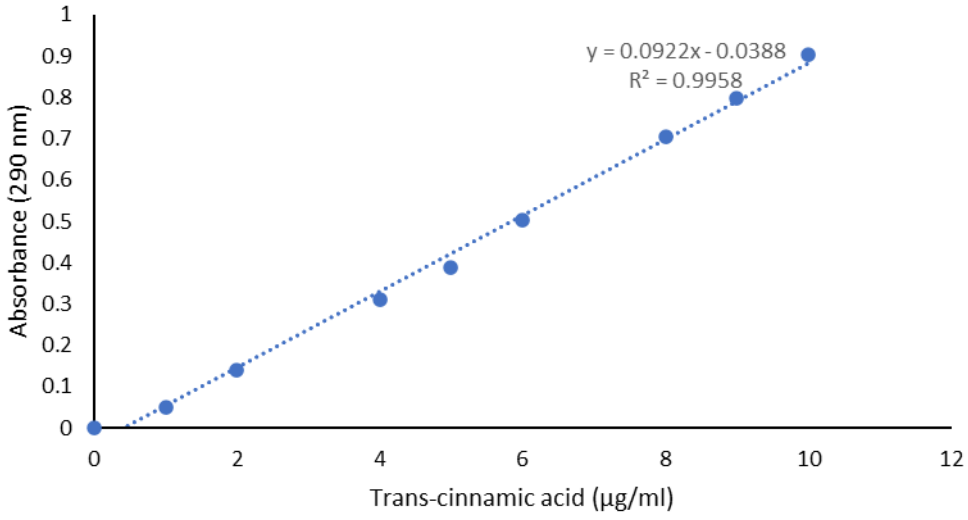


Figure 5. Standard curve for PAL activity.

Hydrogen peroxide activity assay

Hydrogen peroxide activity (H_2O_2) was measured based on Velikova et al. (2000). The liquid nitrogen-ground onion root sample (0.1g) was extracted in 0.1% (w/v)

trichloroacetic acid (2 ml) at 4°C. After centrifugation of the extract at 12,000 rpm for 15 min, the supernatant was used as the enzyme extract. The reaction mixture included 0.05 ml of the enzyme extract, 0.05 ml of potassium phosphate buffer (0.1 M, pH 7.0), and 0.1 ml potassium iodide (1 M), and then the reaction mixture was incubated for 5 min at room temperature. The blank was all reaction mixture without the enzyme extract. The absorbance of the reaction solution was measured at 390 nm by using a Cytation 5 Cell Imaging Multi-Mode Reader (Cytation 5, BioTek, USA). The amount of H_2O_2 was estimated using a standard curve of H_2O_2 and expressed in nmol/g FW of root tissue. The standard curve of H_2O_2 was constructed from known concentrations (0, 5, 10, 20, 30, 40, and 50µM) of H_2O_2 solution (Figure 6).

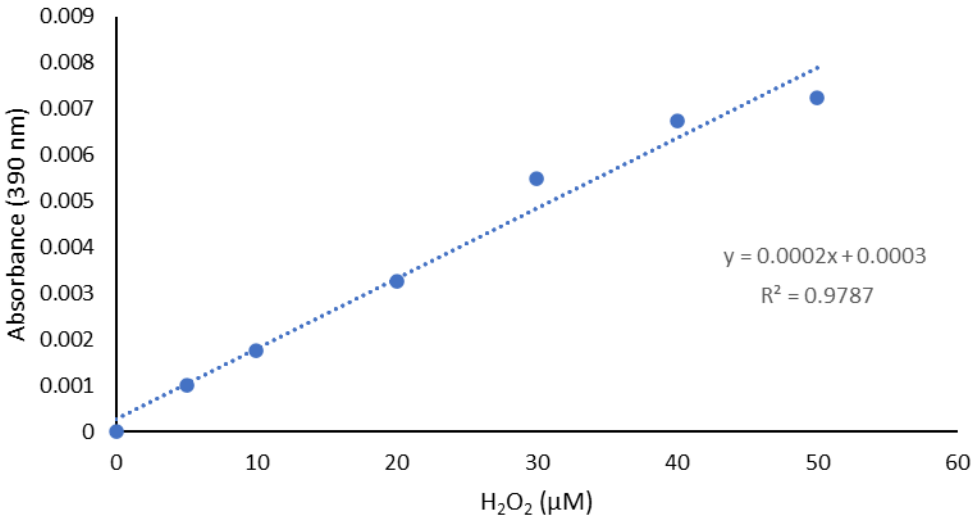


Figure 6. Standard curve for H2O2.

Total phenolic content

Total phenolic content (TPC) was measured according to Graham's method (Graham, 1992). The liquid nitrogen-ground onion root sample (1g) was extracted in 50% methanol (10 ml). The extract was centrifuged at 12,000 rpm for 15 min, and the supernatant was

used as the enzyme extract. The reaction mixture was run for 20 min at room temperature in a test tube with 100 µl supernatant, 900 µl distilled water, 50% Folin–Ciocalteu reagent (500 µl), 20% Na_2CO_3 (1 ml), and 10 ml distilled water. The blank was all reaction mixture without the enzyme extract. After incubation, the colour of the reaction mixture was changed to blue. The absorbance was read at 725 nm by using a Cytation 5 Cell Imaging Multi-Mode Reader (Cytation 5, BioTek, USA). The total phenolic content was determined using a standard curve of gallic acid and expressed in µg of gallic acid per g FW of root tissue. The standard curve of gallic acid was plotted from known concentrations (0, 10, 20, 40, 60, 80, 100, 120, and 140 µg/ml) of gallic acid solution (Figure 7).

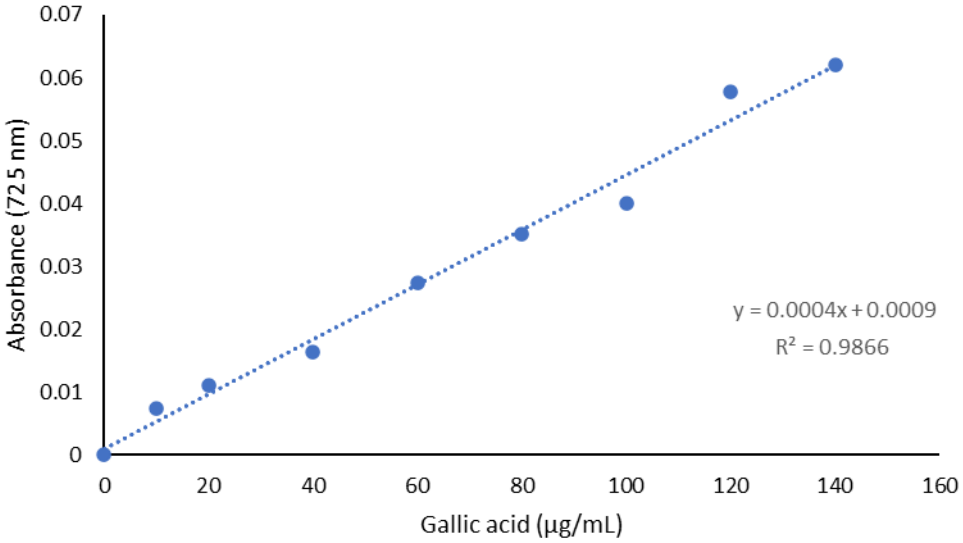


Figure 7. Standard curve for Total phenolic content.

3.8.7 ROOT COLONIALIZATION

Quantification of the pathogen in the roots

The relative amount of pathogen in onion roots was quantified as previously described in Part 3.4.3.

Microscopy

To observe fungal colonization, onion root pieces (3 cm) were stained with lactophenol cotton blue stain and mounted on microscope slides. Microscope slides were overserved using a compound light microscope (Olympus BX63, Japan), and photographs were taken.

3.9 STATISTICAL ANALYSIS

The data were subjected to a one-way analysis of variance (ANOVA) by using Minitab version 19 (Minitab Inc., PA, USA) except for the compatibility test. The means were compared by Tukey's range test at the significance level of $P \leq 0.05$. The compatibility test data were statistically evaluated by repeated measures analysis of variance at the 5% significance level ($\alpha = 0.05$).

CHAPTER 4 : RESULTS

4.1 MORPHOLOGICAL CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF THE FOC ISOLATES

Two isolates of pathogen FOC (Figure 8A&D) were isolated from naturally infected onion plants that were collected from onion fields in Berwick, Nova Scotia. These isolates were identified using morphological features (Figure 8) and sequencing of the ITS region.

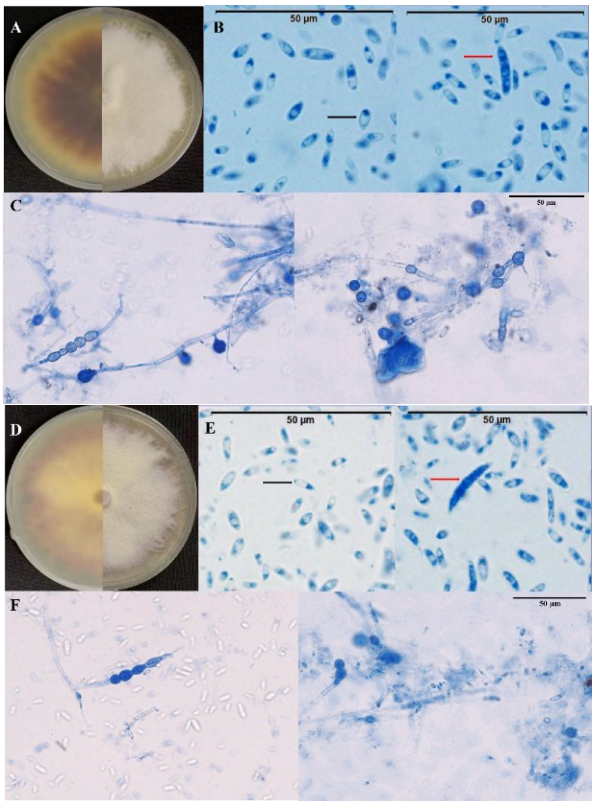


Figure 8. Microscopic characteristics of two FOC isolates (Fa and Fb) from naturally infected onion bulbs. (A) The front and reverse colony morphology of Fa. (B) Microconidia (black arrow) and macroconidia (red arrow) of Fa. (C) Chlamydospores and Mycelium growth of Fa. (D) The front and reverse colony morphology of Fb. (E) Microconidia (black arrow) and macroconidia (red arrow) of Fb. (F) Chlamydospores and Mycelium growth of Fb. Scale bars, B,C,E,F = 50 µm.

Colonies of FOC isolates on PDA medium have dense delicate white aerial mycelia, often with a tinge of purple, on the upper surface. The undersurface may be white to dark purple. Two isolates produce three types of spores: microconidia, macroconidia, and chlamydospores. Microconidia are oval-shaped and single-celled; macroconidia are sickle-shaped and think-walled (usually three- to five-celled); and chlamydospores are spherical, formed in terminal and intercalary positions.

The two FOC isolates were then identified by PCR amplification and sequencing. The genomic DNA of two FOC isolates were amplified with the ITS1 and ITS4 primer, which resulted in amplicons of approximately 500bp (Figure 9). The PCR products were purified and sent for sequencing by ITS1 and ITS4 primer to the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Ontario, Canada). The sequencing results were processed by removing the end sequences that showed bad quality peaks in both forward and reverse sequences. The peaks in both forward and reverse sequences were checked manually and errors were removed. Then the forward and reverse sequenceswere assembled using SeqMan software of the DNASTAR Lasergene software (Figure 10; DNASTAR, Inc., Wisconsin USA). After assembly, the final sequence of Fa (477bp, Appendix I) and Fb (467bp, Appendix G) were obtained. The nucleotide BLAST (BLASTn) analysis of the obtained sequence data showed that both the Fa and Fb isolates from the present study shared more than 99% nucleotide identity with previously reported *Fusarium oxysporum* species (Figure 11). The alignment between Fa and Fb also showed that both sequences are very much similar (99% similarity) to each other (Figure 12). Therefore, combined with the morphological characteristics and ITS sequence similarity analysis, the

two FOC isolates (Fa and Fb) were identified as *Fusarium oxysporum*. However, it was uncertain whether the two FOC isolates were *F. oxysporum* f. sp. *cepae* or not.

Fa and Fb isolates were deposited in the Canadian Collection of Fungal Cultures (DAOMC 252600 and 252601).

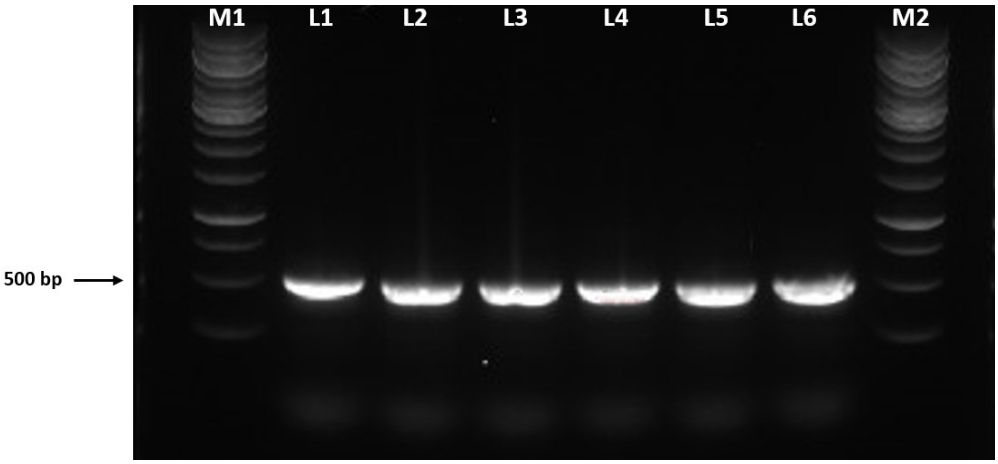


Figure 9. Agarose gel electrophoresis showing the amplified PCR products of ITS set of primers. (Lane M1 and M2 is DNA marker; Lane 1-3 is Fa, and Lane 4-6 is Fb).

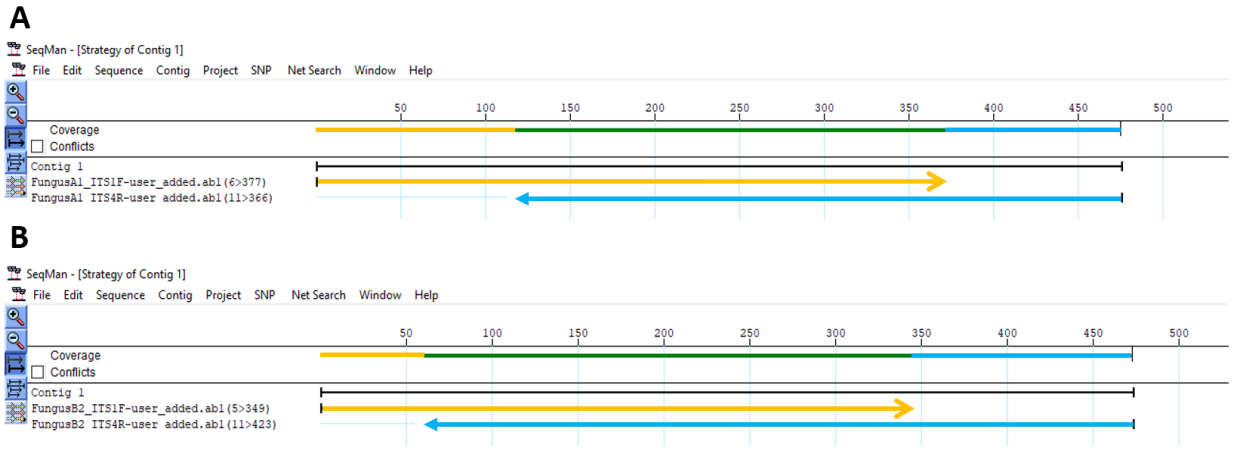


Figure 10. Assembly of forward and reverse sequences for two FOC isolates (Fa and Fb). A) Fa; B) Fb. The common sequence between forward and reverse is shown in green, the forward sequence is shown in yellow, and reverse sequence is shown in blue, as obtained from SeqMan software of DNASTAR.

A

Sequences producing significant alignments Download Select columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fusarium oxysporum isolate FGS1 small subunit ribosomal RNA gene, partial sequence;...	Fusarium ox...	861	861	100%	0.0	100.00%	536	OQ096499.1
Fusarium oxysporum isolate FoxyT11 internal transcribed spacer 1, partial sequence; 5.8...	Fusarium ox...	861	861	100%	0.0	100.00%	527	OP783351.1
Fusarium oxysporum isolate DS1 internal transcribed spacer 1, partial sequence; 5.8S rib...	Fusarium ox...	861	861	100%	0.0	100.00%	515	OP897725.1
Fusarium oxysporum isolate KMDV2 internal transcribed spacer 1, partial sequence; 5.8...	Fusarium ox...	861	861	100%	0.0	100.00%	533	OP897714.1
Fusarium oxysporum isolate KMDV1 internal transcribed spacer 1, partial sequence; 5.8...	Fusarium ox...	861	861	100%	0.0	100.00%	550	OP897707.1
Fusarium sp. isolate 331 small subunit ribosomal RNA gene, partial sequence; internal tr...	Fusarium sp.	861	861	100%	0.0	100.00%	676	OP834780.1
Fusarium oxysporum isolate SP19 small subunit ribosomal RNA gene, partial sequence; i...	Fusarium ox...	861	861	100%	0.0	100.00%	556	OP797662.1
Fusarium oxysporum isolate SP13 small subunit ribosomal RNA gene, partial sequence; i...	Fusarium ox...	861	861	100%	0.0	100.00%	556	OP797659.1
Fusarium oxysporum isolate J1 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Fusarium ox...	861	861	100%	0.0	100.00%	518	OP761896.1
Fusarium oxysporum isolate J2 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Fusarium ox...	861	861	100%	0.0	100.00%	517	OP748375.1

B

Sequences producing significant alignments Download Select columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fusarium oxysporum isolate K_MISO2_5_6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	508	MN452540.1
Fusarium sp. isolate RFE 22 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Fusarium sp.	863	863	100%	0.0	100.00%	521	MT221567.1
Fusarium oxysporum isolate L12_ITS_A02_008.ab1 small subunit ribosomal RNA gene, partial sequence; internal...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	534	MT032671.1
Fusarium oxysporum isolate 2016_D_40_ITS1_D02_005.ab1 internal transcribed spacer 1, partial sequence; 5.8...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	534	MT032612.1
Fusarium oxysporum isolate MR2934 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	517	MN709613.1
Fusarium oxysporum isolate SJ33662 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	542	MN633363.1
Fusarium oxysporum clone 2014_1621 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	522	MN523201.1
Fusarium oxysporum clone 2014_1485 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	514	MN523073.1
Fusarium oxysporum clone 2014_1461 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	512	MN523050.1
Fusarium oxysporum clone 2014_453 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Fusarium oxyspo...	863	863	100%	0.0	100.00%	510	MN522221.1

Figure 11. BLAST analysis top-scored results for the obtained sequence of tested FOC isolates (Fa and Fb) against Genbank database sequences. A) Fa; B) Fb.

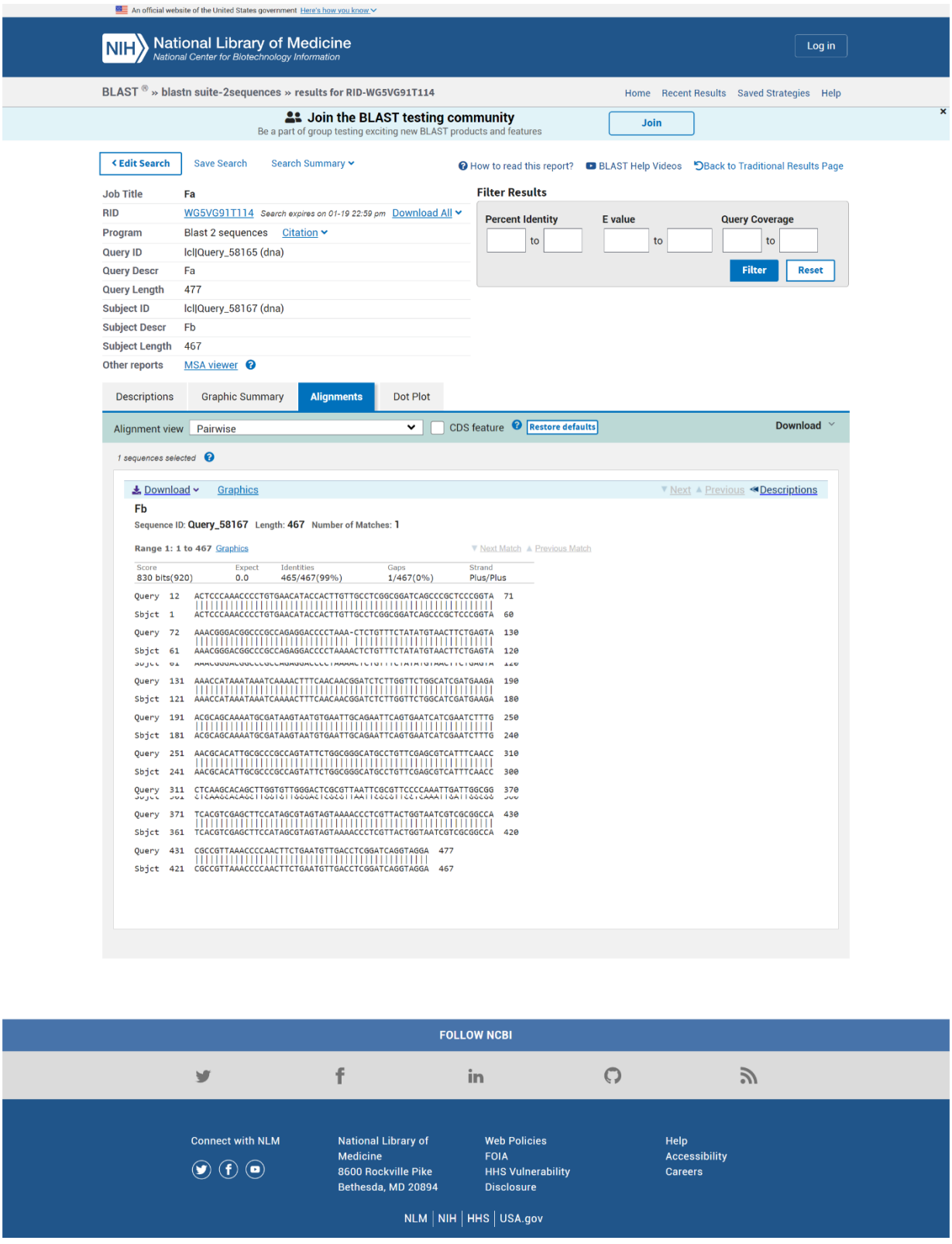


Figure 12. Sequence alignment, produced by BLAST between Fa and Fb.

4.2 PATHOGENICITY TESTS OF ISOLATED FOC

4.2.1 ONION BULB PATHOGENICITY TEST

Fusarium spp. can infect onions in different ways at any stage of their development, and the visual symptoms can be observed in all plant parts, such as leaves, roots, basal stem plate, as well as bulb scales. In the bulb pathogenicity test, one week after inoculation, it was observed that mature onion bulbs developed symptoms, and the control group did not show any symptoms of basal rot or other diseases; thus, the bulbs in the control group were considered as healthy (Figure 13). The Fa inoculated group developed mild symptoms of basal rot, which were brown discoloration and rot formed at the basal plate and inner fleshy scales. The symptoms of the Fb inoculated group were weak and hardly visible (Figure 13).

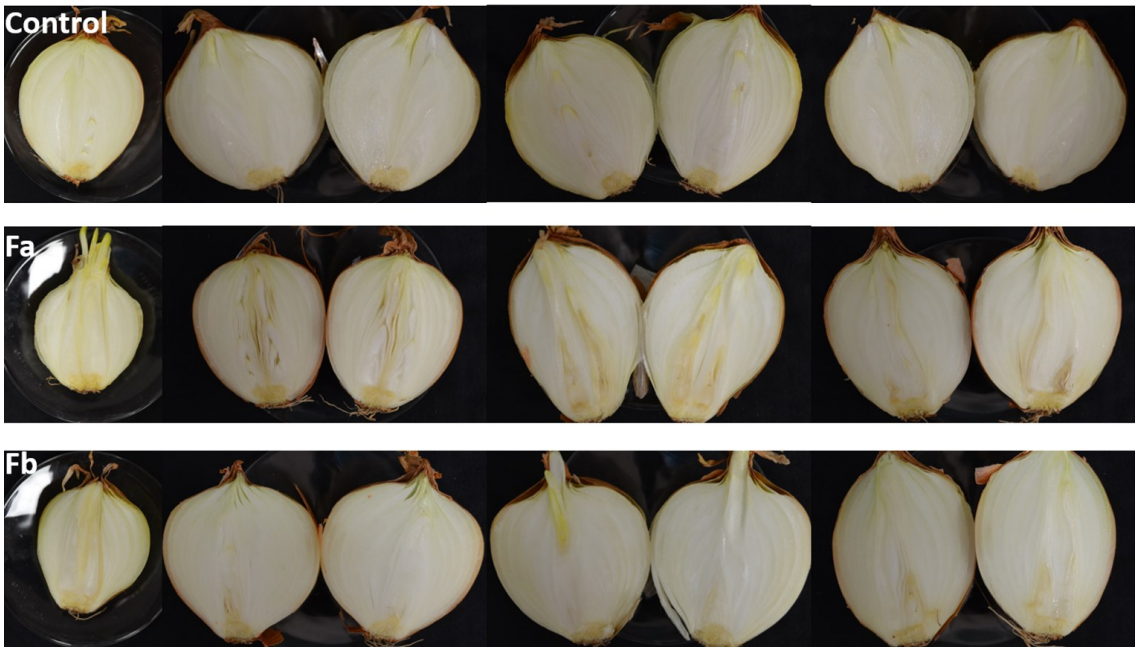


Figure 13. Symptoms of inoculated onion bulbs one week after Fusarium inoculation. Control-SDW.

4.2.2 ONION SEEDLING PATHOGENICITY TEST

We observed similar trends in seed germination percentage for the mycelial disc, spore suspension, and spore-carboxyl methylcellulose (CMC) coating inoculation methods in the preliminary tests (see Appendix A), with the highest seed germination in the control group, followed by the Fa-inoculated group; and the lowest in the Fb-inoculated group. The spore suspension inoculation method was selected for the seedling pathogenicity test, considering that the inoculum density could not be the same by using mycelium disc and spore-CMC coating inoculation methods.

The seedling pathogenicity test showed white mycelia on or near the fusarium-inoculated onion seeds after nine days of incubation, which indicates that the fungus used the onion seeds as a food source. The presence of Fa and Fb apparently reduced the seed germination of onion seeds after being incubated in Petri dishes for 9 days under controlled conditions compared to the control group (Figure 14). Fb caused a lower germination percentage

(52.78%) than Fa (61.67%), but the difference among control, Fa and Fb was not statistically significant.

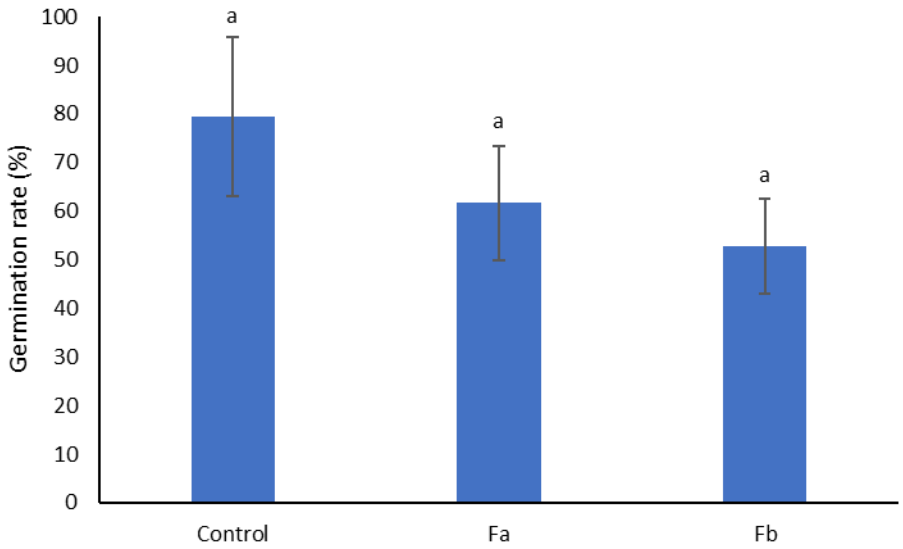


Figure 14. The germination rate of onion seeds inoculation with two FOC isolates (Fa and Fb) and Control (C-SDW). Data were analyzed using One-Way ANOVA. Error bars represent SE (Standard Error). Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

4.2.3 POT PATHOGENICITY TEST

In the preliminary tests (see Appendix B), we observed that none of these methods resulted in the development of expected FBR disease symptoms in onion plants. It was also found that onion plants with cut roots developed symptoms of transplant shock, which were difficult to recover from if we grew them in field soil. Therefore, we decided to use the root dipping method without cutting roots and transplanting onion plants in Pro-mix in this section.

Two fusarium isolates were also tested for their pathogenicity against onion seedlings under greenhouse conditions. Our results showed that neither isolate was able to cause any disease symptoms in the inoculated onion plants. Two isolates tested caused only slight pink roots without any further disease development. For shoot and root biomass (Figure

15), the Fa-inoculated group (2.79g/plant) caused lower shoot biomass than the Fb-inoculated group (3.04g/plant), and the Fb-inoculated group (0.31g/plant) caused lower root biomass than the Fa-inoculated group (0.42g/plant). However, the onion seedlings inoculated with fusarium isolates did not show any significant reduction in the shoot or root biomass compared with the control group. In addition, the lactophenol blue stained onion root longitudinal and cross sections showed fungal invasion in onion root tissues (Figure 16). In order to determine the infections on onion plants caused by the two fusarium isolates, the relative fungal biomass in onion root tissues was evaluated by using a qPCR assay, since no relevant disease symptoms were observed in onion plants one month after pathogen inoculation. The relative amount of Fusarium DNA was detected in the control and two fusarium-inoculated groups by dividing onion DNA into FOC DNA in each sample. The results of quantification showed that the FOC DNA in onion plants infected with Fa and Fb was higher than the control group, and the FOC DNA in the Fa-inoculated group was highest (Figure 17).

Therefore, based on the results of sequencing analysis and three pathogenicity tests, the Fa isolate was selected due to the following reason: (1) Fa caused more obvious disease-related symptoms in the bulb pathogenicity test than Fb; (2) Fa caused the same effect on onion plants as Fb did in the seedlings and pot pathogenicity tests; (3) Fa caused highest relative fungal biomass in onion roots than Fb in the pot pathogenicity test; (4) Both Fa and Fb were identified as *Fusarium oxysporum*.

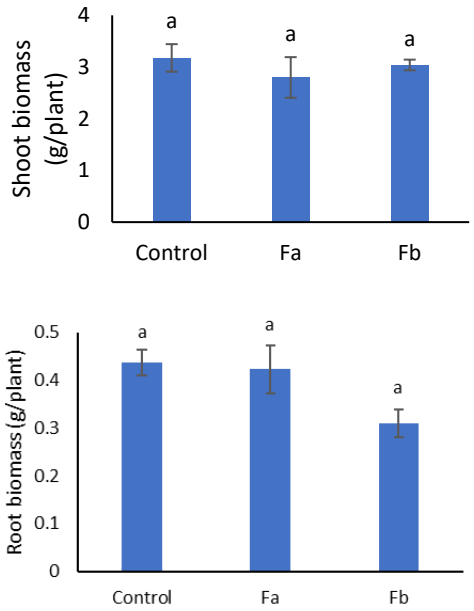
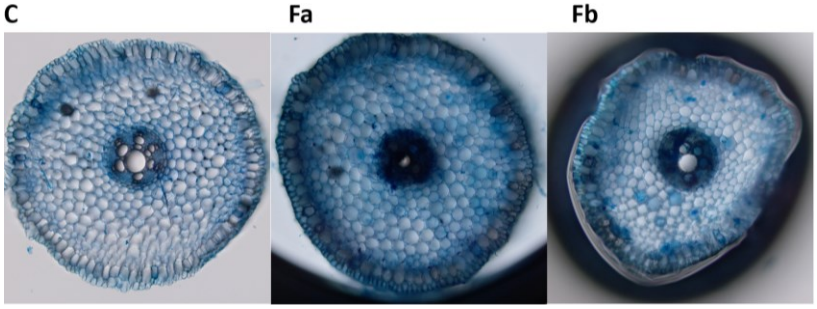


Figure 15. Biomass data (Average fresh shoot weigh-left, average fresh root weight-right) of onion plants inoculated with two FOC isolates (Fa and Fb) and control (SDW). Data were analyzed using One-Way ANOVA. Error bars represent SE. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

a. Onion root cross sections.



b.Onion root longitudinal sections.

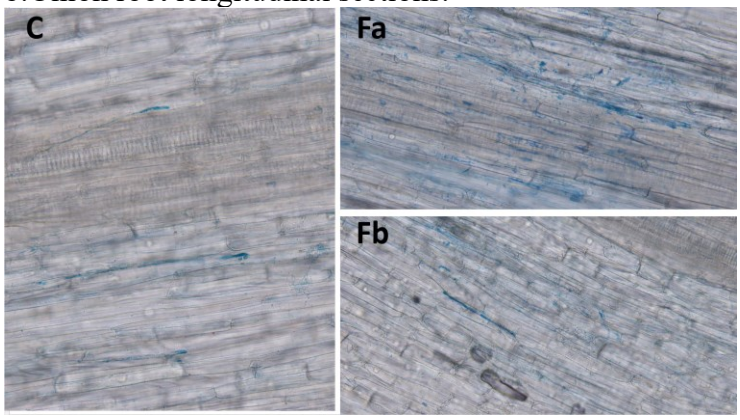


Figure 16. The lactophenol blue-stained onion root sections observed under microscopy.

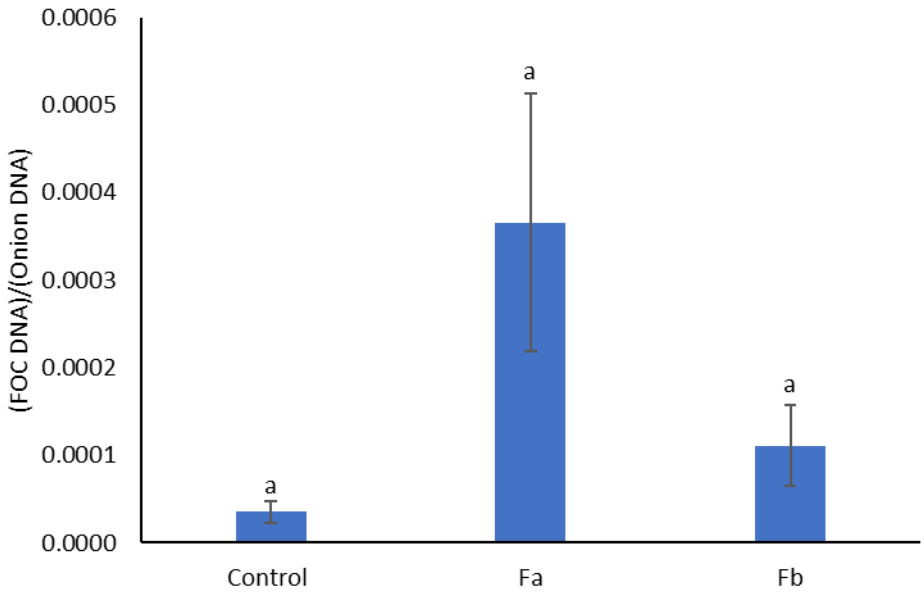


Figure 17. Quantification of the pathogen in the roots of onion plants inoculated with two FOC isolates (Fa and Fb) and control (SDW). Data were analyzed using One-Way ANOVA. Error bars represent SE. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

4.3 EFFECT OF ANE ON THE GROWTH OF THREE PGPR STRAINS

The bacterial growth kinetics of three PGPR strains in liquid broth with different concentrations of ANE were evaluated by measuring the OD_{600} of each PGPR strain after culture from 0 to 48 h. A standard bacterial growth curve in a closed system (Figure 18) includes four phases: lag phase (bacteria adjust to the new medium, and they are preparing for the beginning of a split), log (exponential) phase (bacteria cells are dividing regularly by binary fission and the number of bacteria increases exponentially), stationary phase (the number of dividing cells appears to be equal to the number of dying cells, and dead phase (bacteria lose the ability to divide, resulting in a higher number of dying cells than dividing) (Wang et al., 2015). As shown in Figure 19-21, the growth curves of all PGPR strains

showed an “S” trend. The initial growth of each PGPR in all treatments was similar to each control group, and they all reached the logarithmic phase after 4-6 h. However, during the exponential phase, *P. protegens* CHA0 cultured in 0.1% ANE-modified broth showed higher growth than other treatments (Figure 19), and *B. subtilis* cultured in 0.01% and 0.1% ANE groups were critically lower than the control group (Figure 20), and *E. cloacae* CAL2 cultured in 0.1% and 0.001% ANE-modified broths showed higher growth than others (Figure 21). However, after they reached the stationary phase, there was not much difference between the treatments of each PGPR strain. Analysis of four ANE modified broths of each PGPR by repeated measures showed a significant difference among the treatments over time (Figure 19-21), as expected for differences in production during the logarithmic phase of cultivation.

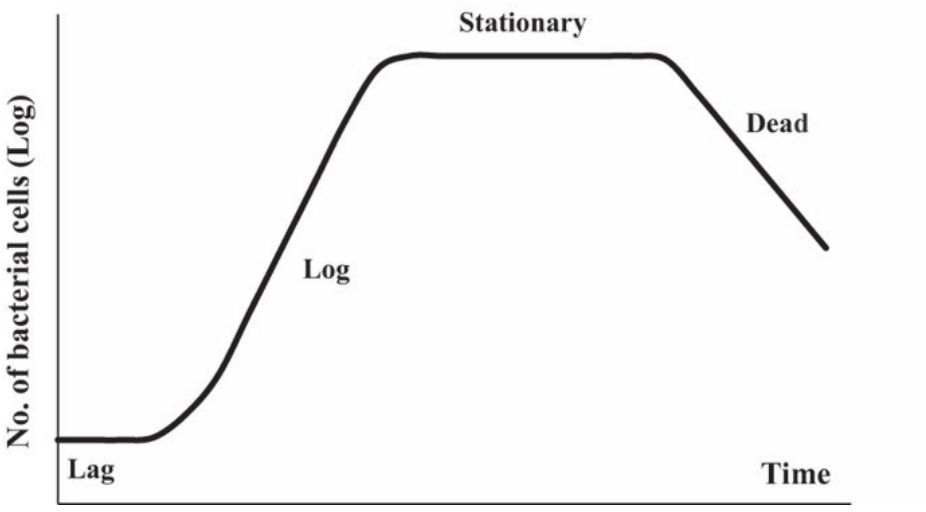


Figure 18. The typical bacterial growth curve in a culture medium (a closed system) (Wang et al., 2015).

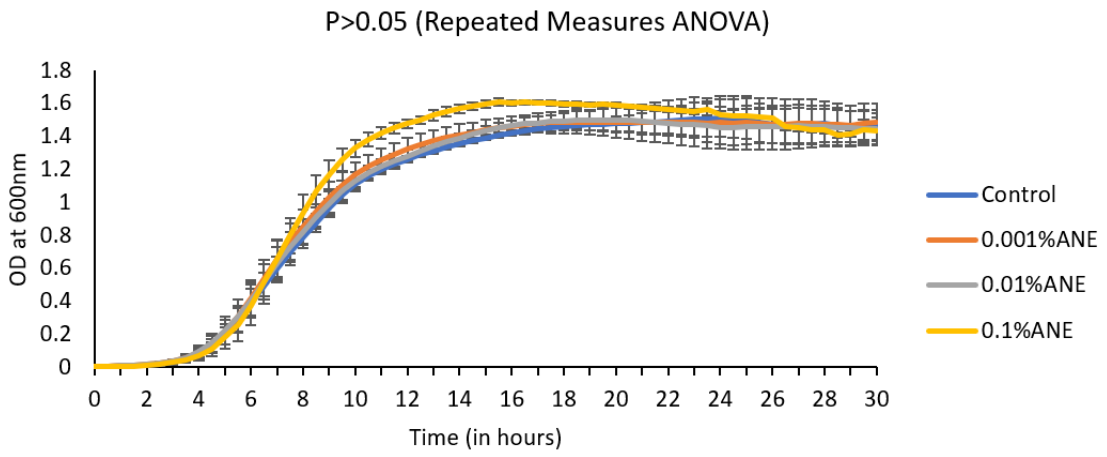


Figure 19. Repeated measures ANOVA test for comparison of liquid media supplemented with four different concentrations of ANE (Control-0%, 0.001%, 0.01%, and 0.1%) for the culture of *P. protegens* CHA0. There was no significant difference among these media and over time (P>0.05). Error bars represent SE.

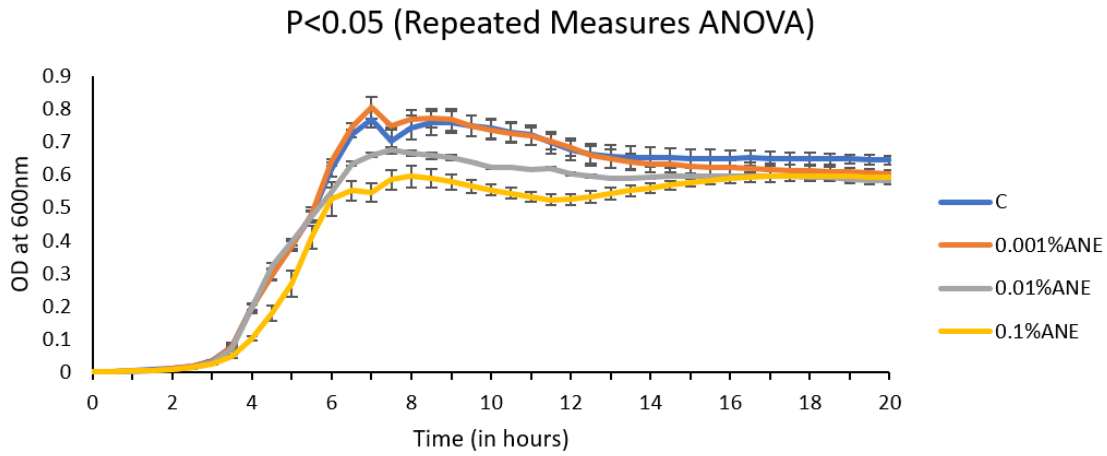


Figure 20. Repeated measures ANOVA test for comparison of liquid media supplemented with four different concentrations of ANE (Control-0%, 0.001%, 0.01%, and 0.1%) for the culture of *B. subtilis*. There was a significant difference among these media and over time (P<0.05). Error bars represent SE.

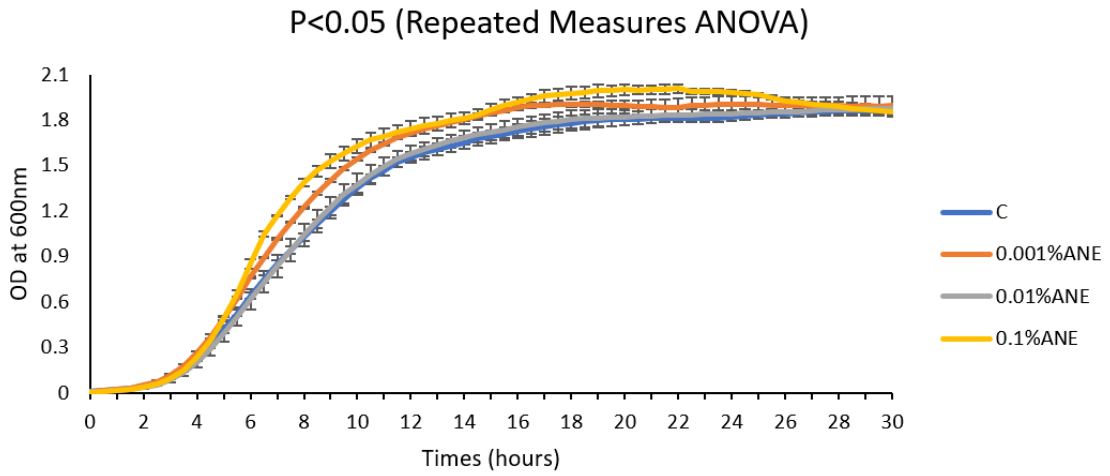


Figure 21. Repeated measures ANOVA test for comparison of liquid media supplemented with four different concentrations of ANE (Control-0%, 0.001%, 0.01%, and 0.1%) for the culture of *E. cloacae* CAL2. There was a significant difference among these media and over time (P<0.05). Error bars represent SE.

4.4 EFFECT OF PGPR AND/OR ANE ON MYCELIAL GROWTH OF FOC

In the present study, only *P. protegens* CHA0 was initially proposed, and different double-culture assay methods were tried (Appendix A) in preliminary experiments, but its antagonistic activity was not effective as expected. Then, *B. subtilis* was considered, but its antagonistic activity was almost similar to that of *P. protegens* CHA0. Therefore, the antagonistic activity of existing PGPR in the laboratory was evaluated, and *E. cloacae* CAL2 was selected finally because it exhibited the highest inhibition effect of mycelial growth (Appendix A).

Three PGPR strains (*P. protegens* CHA0, *B. subtilis*, *E. cloacae* CAL2) were used to study their antagonistic potential against FOC. The results showed that all tested PGPR strains and 0.1% ANE combined with each PGPR exhibited significant pathogen inhibition compared to FOC grown alone in the dual culture assay (Table 8 and Figure 22). Among

these, the strain of *E. cloacae* CAL2 inhibited the mycelial growth of FOC up to 50% compared to the control group. *Pseudomonas protegens* CHA0 (29.7%) and 0.1% ANE combined with *P. protegens* CHA0 (31.1%) exhibited moderate inhibitory activity against the mycelial growth of FOC, and *B. subtilis* (19.3%) and 0.1% ANE combined with *B. subtilis* (17.8%) showed the lowest inhibitory activity (%) against the mycelial growth of FOC. However, there was no significant difference (P>0.05) between each PGPR treatment and the combined treatment of each PGPR and 0.1% ANE (Table 8).

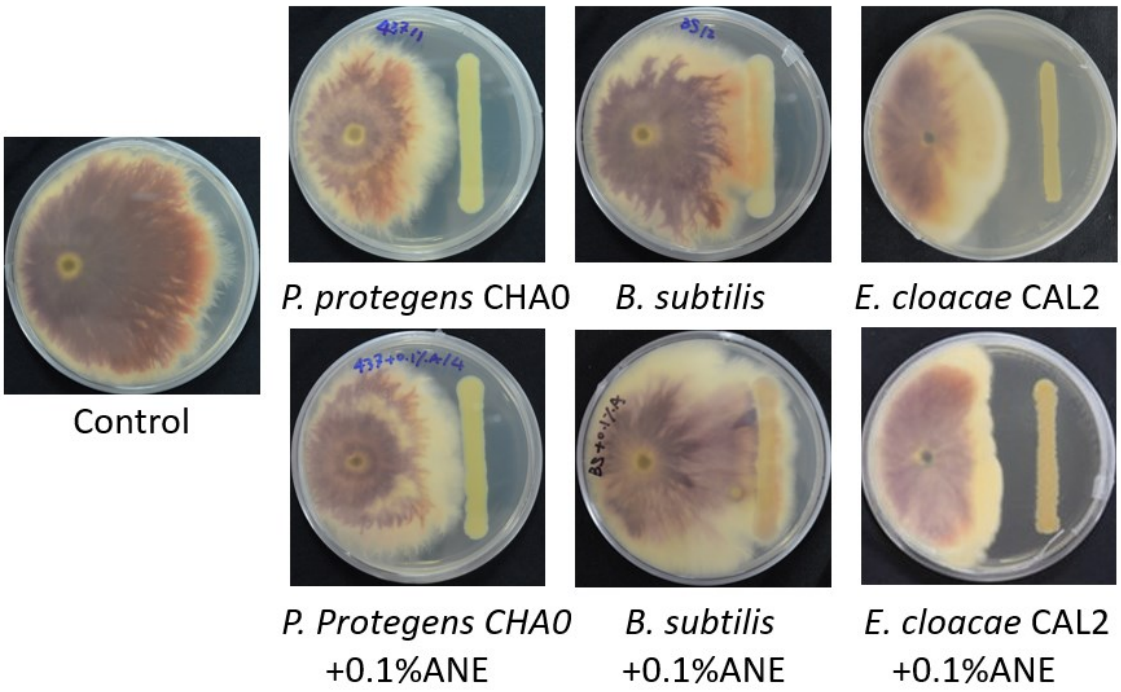


Figure 22. Suppression of mycelial growth of FOC by PGPR strains (*P. protegens* CHA0, *B. subtilis*, and *E. cloacae* CAL2) under *in vitro* conditions by using a dual culture test on PDA media.

Table 8. Dual culture assay of each PGPR with/without ANE against the pathogen mycelial growth. a. *P. protegens* CHA0; b. *B. subtilis*; c. *E. cloacae* CAL2. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

a.

Treatment	Inhibition%±SE	
CHA0+0.1% ANE	31.1±3.4	a
CHA0	29.7±2.9	a
Control	0.0	b

b.

Treatment	Inhibition%±SE	
BS+0.1% ANE	19.3±3.1	a
BS	17.8±2.0	a
Control	0.0	b

c.

Treatments	Inhibition%±SE	
CAL2	54.1±1.1	a
CAL2+0.1% ANE	53.6±1.1	a
Control	0.0	b

4.5 EFFECT OF PGPR AND/OR ANE ON SPORE GERMINATION OF FOC

The ability of three PGPR strains to inhibit spore germination of FOC was evaluated. The results were similar to the dual culture assay (Figure 23). It was observed that all three PGPR strains and combined treatments showed a significant inhibitory effect on spore germination of FOC compared to the control group of SDW (Figure 23). *E. cloacae* CAL2 (10.9%) and the combined treatment of 0.1% ANE and *E. cloacae* CAL2 strongly inhibited the spore germination of FOC. *P. protegens* CHA0 (36.7%) and the combined treatment of 0.1% ANE and *P. protegens* CHA0 (30.6%) were the next most effective, and *B. subtilis* (50.0%) and the combined treatment of 0.1% ANE and *B. subtilis* (40.6%) showed the lowest inhibitory activity against the spore germination of FOC. ANE treatment showed no effect on the spore germination of FOC, and there was no significant difference ($P>0.05$)

between each PGPR treatment and the combined treatment of each PGPR and 0.1% ANE (Figure 23).

Based on the results of *in vitro* tests, *E. Cloacae* CAL 2 was selected to control onion FBR disease under greenhouse conditions as it caused the highest antifungal activity under *in vitro* studies, and it was found to be compatible with ANE. Therefore, *E. Cloacae* CAL 2 and Fa isolate of FOC were used in the greenhouse experiment.

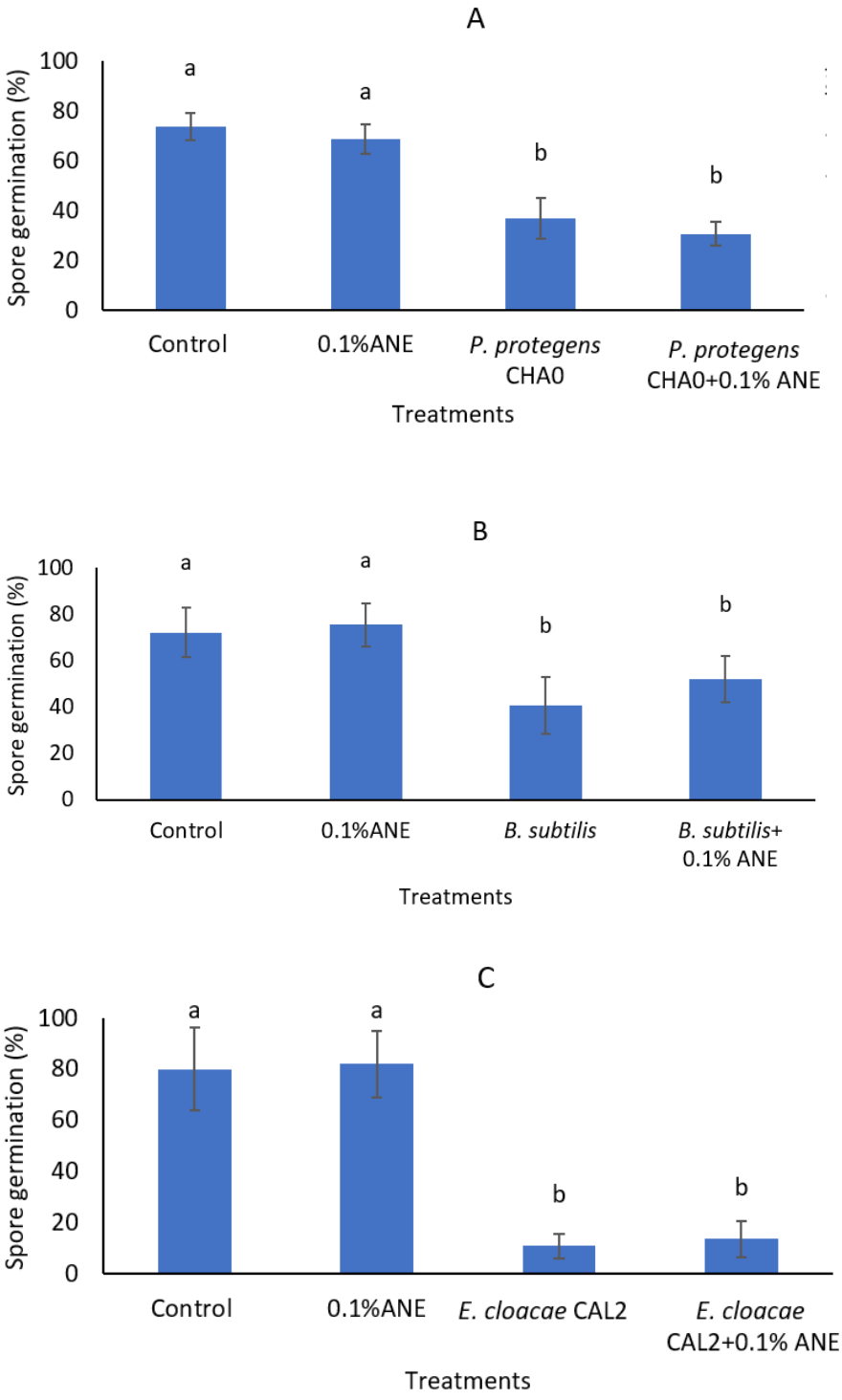


Figure 23. Inhibitory effect of each PGPR and ANE on spore germination of the pathogen. A. *P. protegens* CHA0; B. *B. subtilis*; C. *E. cloacae* CAL2. Data was analyzed using One-Way ANOVA. Error bars represent SE. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

4.6 EFFECT OF *E. CLOACAE* AND/OR ANE ON ONION FBR DISEASE UNDER GREENHOUSE CONDITION

4.6.1 EFFECTS OF *E. CLOACAE* AND/OR ANE ON DEFENCE-RELATED ENZYMES AND BIOCHEMICAL ACTIVITIES

PO and PAL are important defence-related enzymes involved in plant disease resistance. When plants are attacked by pathogens or treated by microbial/non-microbial elicitors, they rapidly produce reactive oxygen species (ROS) against pathogen infestation. PO catalyzes ROS, mediates lignification, and is also involved in the production of anti-microbial metabolites (phytoalexin) (Almagro et al., 2009). PAL is a key enzyme involved in the phenylpropanoid pathway, which is important in the synthesis of various phenolic substances that are related to plant structural defences (Hahlbrock & Scheel, 1989; Verberne et al., 1999). PAL is also involved in the biosynthesis of SA, which is an important signal that regulates plant resistance (Kim & Hwang, 2014).

To determine the activity of PO and PAL and the production of H_2O_2 and total phenolic content in Fusarium inoculated and non-inoculated plants, which were treated with 0.5% ANE (T2), CAL2 (T3), 0.5% ANE+CAL2 (T4), and SDW (T1-control group). Root tissues were collected at different time points (24, 48, and 72 hours) after Fusarium inoculation. The results (Figure 24) showed that the interaction between treatments and time points did not significantly affect the level of PO ($P>0.05$) and PAL ($P>0.05$). Treatments did not significantly affect the activity of PO ($P>0.05$) and PAL ($P>0.05$), and there was also no significant difference between the time points ($P>0.05$ for PO, $P>0.05$ for PAL). Similarly, there was no significant interaction effect between treatments and time points on H_2O_2 ($P>0.05$) and TPC ($P>0.05$). Treatments did not significantly affect H_2O_2 ($P>0.05$) and

TPC (P>0.05), but there were significant differences between the time points in the production of H_2O_2 (P>0.05) and TPC (P>0.05).

4.6.2 EFFECTS OF *E. CLOACAE* AND/OR ANE ON ONION BIOMASS

Onion biomass was measured 30 days after inoculation and presented in terms of average shoot and root weight (Figure 25). The highest average shoot weights were observed in T3 without pathogen inoculation, followed by T3 with pathogen inoculation, T1 without pathogen inoculation, and T1 with pathogen inoculation. The highest average root weights were observed in T4 with pathogen inoculation, followed by T3 with pathogen inoculation, T1 without pathogen inoculation, and T3 without pathogen inoculation. We also observed that the average shoot weights were lowest in T2 with pathogen inoculation, T4 with pathogen inoculation, and T2 with pathogen inoculation; and the average root weights were lowest in T2 with pathogen inoculation, T2 without pathogen inoculation, and T4 without pathogen inoculation. However, there was no difference between all treatments in the average shoot and root weight.

4.6.3 EFFECTS OF *E. CLOACAE* AND/OR ANE ON FUNGAL BIOMASS IN INFECTED PLANTS

Since inoculated onion plants did not develop any disease symptoms one month after pathogen inoculation, disease quantification was assessed indirectly by quantifying the relative amount of fungal DNA in onion roots. The results (Figure 26) showed that the fungal DNA quantity in the T2 treatment was significantly higher than in other treatments, and the fungal DNA quantity in T3 and T4 treatments were higher than the control group but not statistically significant.

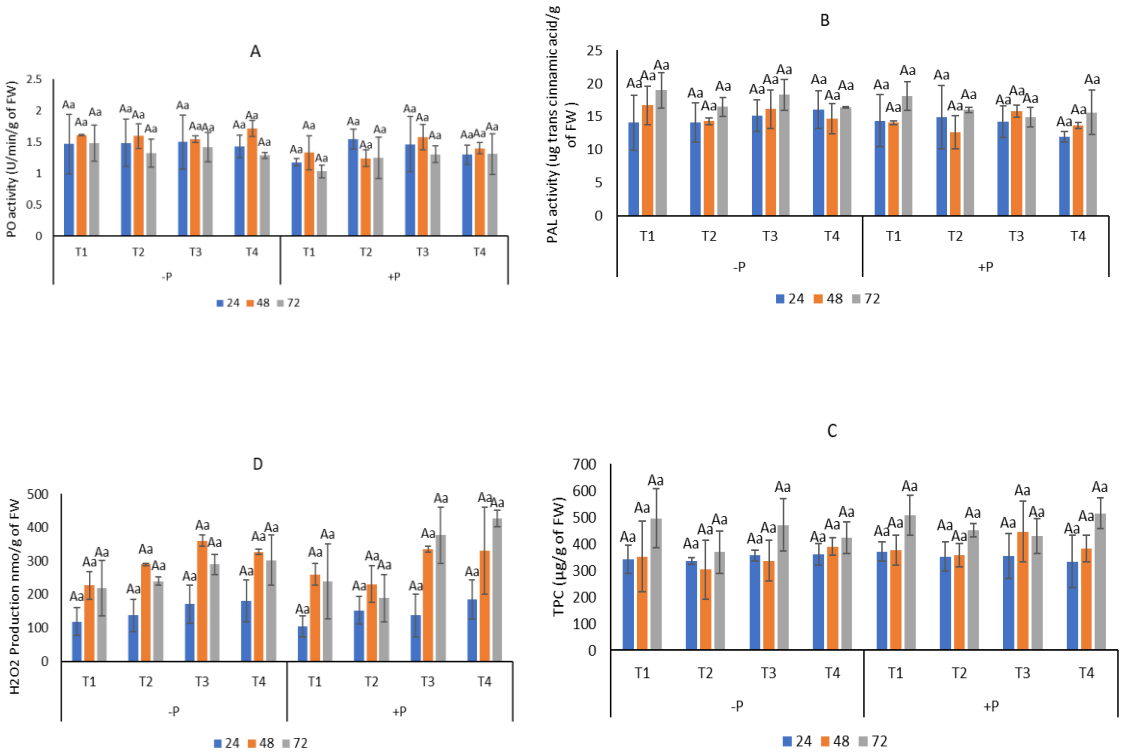


Figure 24. Peroxidase (PO) and Phenylalanine ammonia lyase (PAL) activity, and production of hydrogen peroxide (H2O2) and Total phenolic content (TPC) in onion roots at 24 h, 48 h, and 72 h after the inoculation of FOC. A. PO activity; B. PAL activity. C. H_2O_2 production; D. TPC production. T1 (control group-SDW), T2 (0.5% ANE), T3 (CAL2), and T4 (0.5% ANE+CAL2). -P: without FOC inoculation, +P with FOC inoculation. Data were analyzed using One-Way ANOVA. Error bars represent SE. Lowercase letters imply statistical differences between experimental time points within each treatment ($P \leq 0.05$; Tukey's multiple comparisons); and capital letters indicate statistical differences among the different treatments for the same time point ($P \leq 0.05$; Tukey's multiple comparisons).

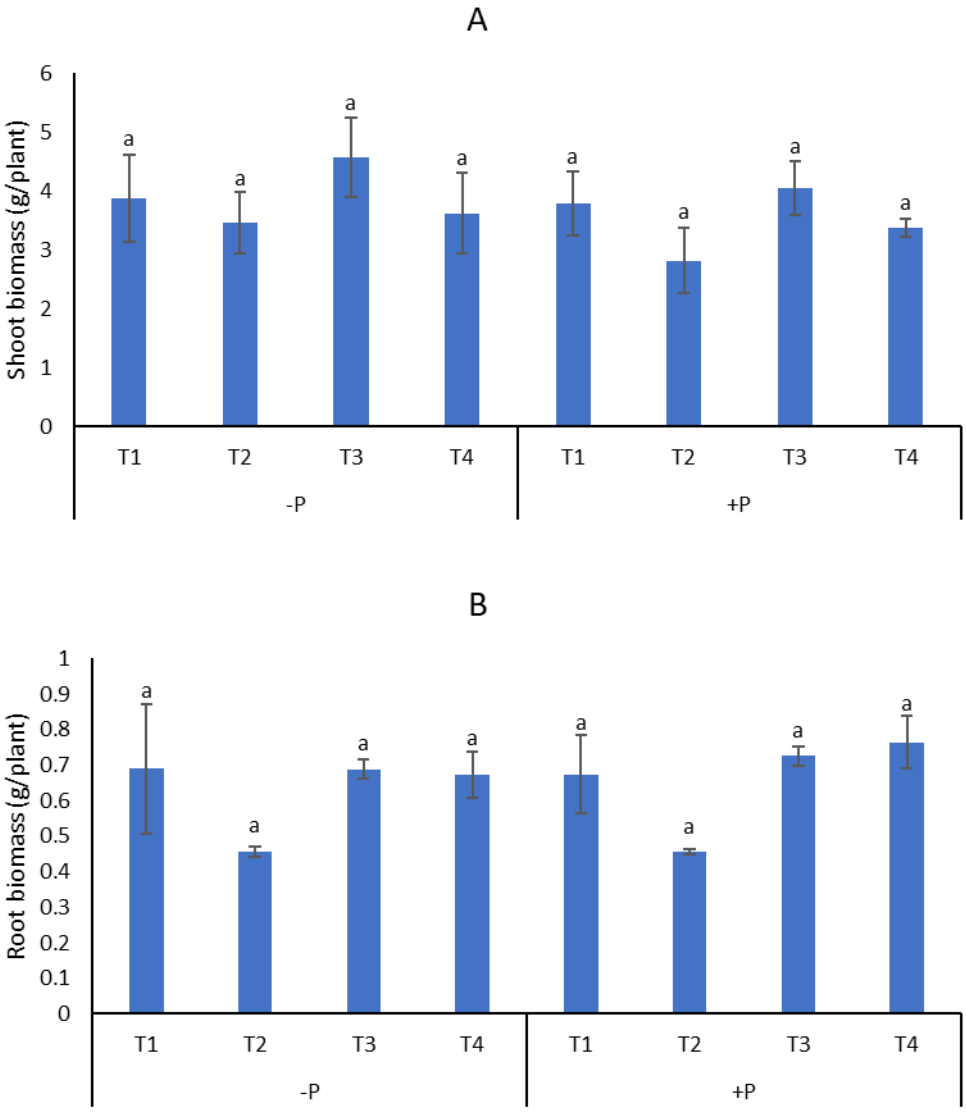


Figure 25. Biomass data (Average fresh shoot weigh-A, average fresh root weight-B) of onion one month after the inoculation with FOC. T1 (control group-SDW), T2 (0.5% ANE), T3 (CAL2), and T4 (0.5% ANE+CAL2). -P: without FOC infection, +P with FOC infection. Data were analyzed using One-Way ANOVA. Error bars represent. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

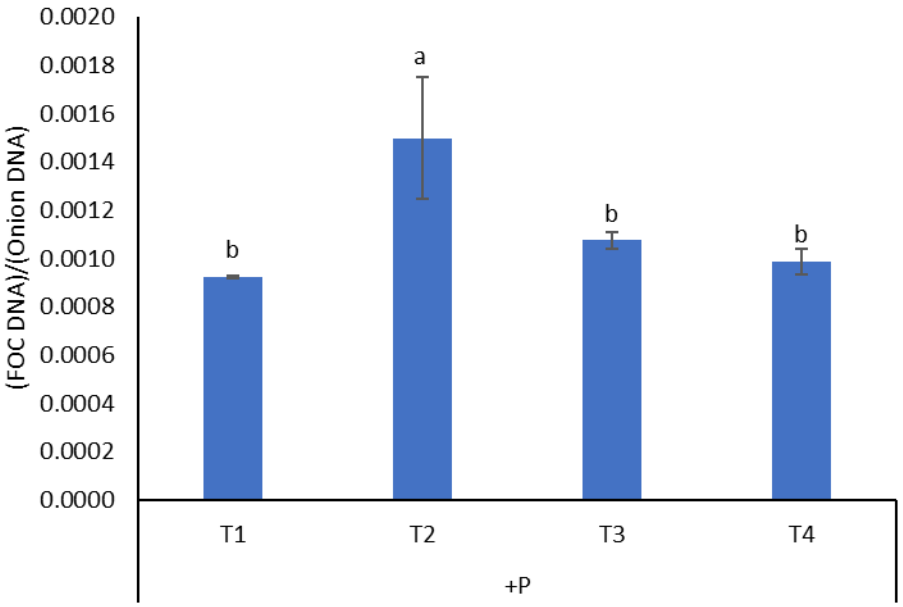


Figure 26. Quantification of the pathogen in the roots of onion plants one month after the inoculation with FOC. T1 (control group-SDW), T2 (0.5% ANE), T3 (CAL2), and T4 (0.5% ANE+CAL2). Data were analyzed using One-Way ANOVA. Error bars represent SE. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

CHAPTER 5 : DISCUSSION

Currently, global agricultural development is facing various problems and challenges, such as rapid population growth, which drives increasing demand for food, climate change, and depletion of natural resources (land and water). Global onion production faces serious yield and economic losses due to Fusarium basal rot (FBR) caused by *Fusarium oxysporum* f. sp. *cepae* (FOC) Bunbury-Blanchette & Walker, 2019). This fungus is a soil-borne pathogen that is very difficult to control because it produces chlamydospores that can survive in the soil for a long time (Cramer, 2000; Özer et al., 2004). Currently, there are no completely FBR-resistant cultivars, crop rotation and synthetic fungicides are not completely effective, and the use of synthetic fungicides can raise other environmental problems (Le & Haesaert, 2021). Therefore, more and more attention has been given to biological control as an ecologically friendly and promising method for plant pathogen control.

Based on the morphological characteristics and sequencing results, the two FOC isolates we isolated from naturally infected onion bulbs were identified as *F. oxysporum*. It is still uncertain whether the two FOC isolates are *F. oxysporum* f. sp. *cepae*. However, several studies have indicated that not only *F. oxysporum* f. sp. *cepae* can cause onion FBR disease. For example, *F. proliferatum*, *F. oxysporum* f. sp. *cepae*, *F. acutatum*, and *F. anthophilium* caused onion FBR in northern Israel (Kalman et al., 2020). *F. oxysporum*, *F. solani*, and *F. proliferatum* were found as causal agents of onion FBR in Vietnam (Le et al., 2021). Recently, *F. falciforme* and *F. brachygibbosum* have been reported as two new causative agents of onion FBR disease in Mexico (Tirado-Ramirez et al., 2021).

Ascophyllum nodosum is a brown seaweed that contains a variety of bioactive compounds (e.g., polysaccharides, growth regulators, and polyphenols) and has been reported to promote plant growth and productivity (Shukla et al., 2019). Compared with synthetic fungicides, seaweed extracts are highly organic and have the characteristics of being bio-degradable, non-toxic, and eco-friendly (Ali et al., 2021b). Previous studies have shown that *A. nodosum* extract contains bioactive elicitors that can induce plant resistance against various diseases (Bajpai et al., 2019; Gunupuru et al., 2019; Patel et al., 2020; Shukla et al., 2019). Plant growth promoting rhizobacteria (PGPR), a group of free-living, root-colonizing soil bacteria, are another type of biological control agent that also has promising prospects in the control of plant diseases. PGPRs can control plant diseases through different mechanisms such as competition, antibiotics and lytic enzyme production, and induction of plant resistance (Basu et al., 2021; Goswami et al., 2016; Vejan et al., 2016). Previous studies have reported the effectiveness of many PGPR (such as *Pseudomonas* spp., *Bacillus* spp., and *Enterobacter* spp.) as biocontrol agents against various pathogens (Ashrafi et al., 2021; Bellameche et al., 2021; Hafez et al., 2020; Kim et al., 2020; Kumbar et al., 2019; Mohamed et al., 2020). Therefore, the present study evaluated the effectiveness of *A. nodosum* extract (ANE) and PGPR alone or their combination against FOC under *in vitro* and greenhouse conditions.

The first objective of the present study was to determine the antagonistic activity of ANE and PGPR alone or their combination against FOC under *in vitro* conditions. The results of the compatibility test showed that *P. protegens* CHA0 and *E. cloacae* CAL2 were compatible with ANE, but the addition of ANE had a negative effect on the growth of *B. subtilis* (Figure 19-21). Several studies indicated that the compatibility of the combination

of introduced biocontrol agents is critically essential for establishing better and more consistent disease suppression, and the combination of PGPR or PGPR with non-microbial products may have higher adaptability and exhibit biocontrol activity against multiple pathogens than a single PGPR under various environmental conditions in the field (Janisiewicz, 1996; Janisiewicz & Bors, 1995; Latha et al., 2009; Liu et al., 2017; Raaijmakers et al., 1995; Sundaramoorthy et al., 2012). Shoaib et al. (2018) reported that comparing the individual application of *Trichoderma* spp. or dry leaf biomass of *Azadirachta indica* L., the combined application had a better effect on decreasing charcoal rot disease incidence and improving the growth of cowpea plants. Chaves-Gómez et al. (2021) also reported that the combined treatment of *Trichoderma virens* with *Bacillus velezensis*, and *T. virens* or *B. velezensis* with chitosan exhibited the highest efficiencies in the management of vascular wilt disease caused by *F. oxysporum* in cape gooseberry plants. In the present study, the treatment of each PGPR alone and combined with ANE inhibited the mycelial growth and spore germination of FOC, among which *B. subtilis* had the least inhibitory effect on FOC, and *E. cloacae* CAL2 showed the greatest inhibitory effect on FOC (Figure 22&23, Table 8). However, ANE did not show any inhibitory activity against FOC. The inhibitory effect of each PGPR strain against FOC might be due to the presence of bacterial cells and/or the production of antibiotic metabolites. Previous studies have reported the antifungal activity of diffusible and volatile compounds produced by several strains of *Pseudomonas*, *Bacillus,* and *Enterobacter* spp. (Chaouachi et al., 2021; Gong et al., 2019; Siahmoshteh et al., 2018; Wallace et al., 2017; Wang et al., 2021; Zhang et al., 2020). The direct antifungal activity of seaweed extracts has been reported, but the antimicrobial efficiency of seaweed extracts also depends on the algal species as well as

the type of solvents used for extraction (Mohamed & Saber, 2019; Mohy El-Din & Mohyeldin, 2018; Radhika et al., 2012). Mohy El-Din & Mohyeldin (2018) investigated the antifungal activity of four brown seaweeds, which were collected in different seasons and extracted with different solvents. The results showed that their methanolic extracts exhibited better antifungal activity than other solvent extracts and the best antifungal activity in spring. However, another similar study reported that among the different extracts of brown seaweed *Hormophysa cuneiformis*, only its chloroform extract has higher antifungal activity against all tested fungi than other extracts (Mohamed & Saber, 2019). Somai-Jemmali et al. (2020) reported that an *A. nodosum*-based product (Dalgin Active®) did not significantly inhibit the mycelial growth and spore germination rates of *Zymoseptoria tritici* under *in vitro* conditions, while its foliar application inhibited sporulation on the leaf surface, decreased fungal cell wall-degrading enzymes (β-1,4-endoxylanase and β-1,3-endoglucanase) and protease activities as well as activated defence mechanisms in plants. However, we found no differences between the combination of PGPR and ANE and their individual treatment, which means although ANE enhanced the growth of *P. protegens* CHA0 and *E. cloacae* CAL2, but it has no effect on the antifungal activity of *P. protegens* CHA0 and *E. cloacae* CAL2. Different from our findings, the combined application of *Trichoderma viride*, *Pseudomonas fluorescens,* and Zimmu (*Allium cepa* L. *x Allium sativum* L.) leaf extract resulted in greater inhibition of mycelial growth of *Pythium aphanidermatum* than the individual use of *T. viride* or *P. fluorescens* (Muthukumar et al., 2010). Similarly, the combination treatment of *P. fluorescens* (Pf1), *P. fluorescens* (Py15), *B. subtilis* (Bs16), and Zimmu leaf extract caused the highest inhibition of mycelial growth in *A. solani* (Latha et al., 2009). Therefore, the improvement of the

antimicrobial ability of PGPR may rely on the strain of PGPR and the type of another biocontrol agent.

The second objective of the present study was to investigate the effect of ANE and PGPR alone or their combination in the control of onion FBR disease caused by FOC under greenhouse conditions. The results of *in vitro* experiments suggested that *E. cloacae* CAL2 strain and its combination with ANE showed a higher inhibitory effect on FOC than the other two strains (Figure 22&23, Table 8). However, in our greenhouse experiment, it was observed that the treatment of ANE, *E. cloacae* CAL2 strain, and their combination did not significantly increase defence-related enzymes (PO and PAL) activities and TPC, but slightly increased the production of H_2O_2 in the presence and absence of challenge (Figure 24). The results from previous studies are not in agreement with our results. Previous studies reported that ANE treatment significantly induced plant resistance by activating defence-related enzymes such as PO, PAL, and PPO and accumulating H_2O_2 and phenolic compounds (Bajpai et al., 2019; Gunupuru et al., 2019; Patel et al., 2020). Similarly, the application of *E. cloacae* PS14 effectively induced plant resistance which is associated with enhanced defence-related enzymes (PO, PPO, and lipoxygenase) activities and increased total phenol and SA contents in potato plants (Mohamed et al., 2020). Defence-related enzymes play a significant role in plant disease resistance, PO is involved in the hypersensitive response, lignification, and phytoalexin production (Almagro et al., 2009), and PAL is involved in the phenylpropanoid pathway that leads to the production of various phenolic compounds (Hahlbrock & Scheel, 1989; Verberne et al., 1999). The increase in PO activity leads to the production of ROS, which is known as the oxidative burst (Prasannath, 2017). The H_2O_2 acts as a direct antimicrobial agent to kill or slow the growth

of pathogens. Peroxidase and H_2O_2 also participate in the process of lignification to reinforce the cell wall and limit the invading pathogens in host tissues (Almagro et al., 2009; Obianom et al., 2019). The reason for these inconsistent results may be related to the strain of PGPR, the plant species, and the environmental condition. Van Loon (2007) has pointed out that *Arabidopsis* responds differently to different PGPRs, even though all these PGPR strains are capable of triggering ISR in this species. It has also been indicated that the *Bacillus* strains were more responsive to corn than to soybean or wheat (Akinrinlola et al., 2018; Tilak & Reddy, 2006). Dual culture assay test has been used as a useful method to determine the antagonistic ability of microbes on agar plates, but it cannot determine other biocontrol properties of PGPRs, such as hyperparasitism, predation and niche competition (Kim et al., 2020). Even though the greenhouse is a controlled environment, it is still more complicated than *in vitro* conditions. Several studies have reported the inconsistent performance of biocontrol agents in both *in vitro* and *in vivo* experiments (Besset-Manzoni et al., 2019; Boulahouat et al., 2022; Comby et al., 2017). Boulahouat et al. (2022) found that the isolated *B. paralicheniformis* strain 260, which expressed a low inhibition percentage of fungal mycelia under *in vitro* conditions, provided significant protection (85%) of palm seeds under *in vivo* conditions against *Fusarium oxysporum* f.sp. *albedinis*. The isolated strains (84 and 333) exhibited a higher inhibition rate of fungal mycelia under *in vivo* conditions, while they showed only moderate protection (about 60%) of palm seeds against *Fusarium oxysporum* f.sp. *albedinis* under *in vivo* conditions (Boulahouat et al., 2022). It was also mentioned that this might be due to different bacteria differ in their conolization ability of plant roots, and the endophyte strains exhibited better biocontrol effect under *in vivo* tests as it characterized by strong root colonization

capabilities (Boulahouat et al., 2022). The biocontrol and plant promotion ability of PGPRs could be affected by the type, moisture, pH and organic matter of soil (Basu et al., 2021; Mahanty et al., 2017). Nguyen et al. (2019) suggested that air and soil temperature affect PGPR efficiency when exposed to temperate field conditions. They found that *Bacillus velezensis* application was able to increase the grain yield in wheat when the temperatures are optimal in 2014, but when there was low temperature during and after inoculation of *B. velezensis* in 2015, no significant increase in grain yield was observed. Therefore, they concluded that the low temperature in the field could affect the PGPR efficiency (Nguyen et al., 2019). In addition, the amount and the time-course of the increase in the defence-related enzymes depending on the elicitor and host plant. For example, Bajpai et al. (2019) observed that defence-related enzymes (PAL, PO, and PPO) were induced 72h post-inoculation and continued to increase to 120h in ANE treated strawberry plants. The application of *T. asperellum* T34 significantly increased PO activity on the 8th day after inoculation and PPO and lipoxygenase on the 2nd day after inoculation in potato plants. *E. cloacae* PS14 treatment significantly increased PO activity on the 4th day after inoculation and PPO and lipoxygenase on the 2nd day after inoculation in potato plants (Mohamed et al., 2020).

The results of the present study showed that one month after the inoculation of FOC, the tested bioformulations did not exhibit a significant effect on the growth parameters of both pathogen-inoculated and uninoculated onion seedlings in terms of shoot and root biomass as compared to the control (T1-SDW) treatments (Figure 25). We also observed that shoot and root biomass in ANE-treated onion seedlings in both with and without pathogen inoculation groups were lower than those in other treatments (Figure 25), which may be

due to the concentration of ANE being too high is toxic to plants and inhibits plant growth. Hernández-Herrera et al. (2014) indicated that the applications of liquid seaweed extracts of *Caulerpa sertularioides* and *Sargassum liebmannii* at a higher concentration (1.0%) inhibited the ability of the tomato seeds to imbibe water and reduced the germination and growth (plumule length and radicle length) of the seeds. They pointed out that the negative effects of seaweed extracts on the plants may be due to the high salinity of the extracts. Salinity has many adverse effects on plant development, such as germination, growth, and photosynthesis. Salinity stress inhibits photosynthesis, disturbs cellular ion homeostasis, causes membrane damage, and finally results in a reduction in plant biomass (e.g., the weight and length of roots and shoots) (Arif et al., 2020; Hao et al., 2021). Hamouda et al. (2022) also reported that higher concentrations of seaweed liquid fertilizer inhibited morphological measurements (e.g., germination percentage, total plant height, and total dry mass) of wheat seedlings and slowed the mitotic index rate of wheat root cells. Previous research indicated that high concentrations of seaweed extracts might contain high levels of minerals (e.g., Fe, Zn, Mn, and Cu), phenols, and alkaloids. These minerals can damage plant cells in several ways, which was reflected in inhibited enzymatic activities, nutrient uptake, DNA replication, gene expression, and cell division, depolarized cell membrane, increased membrane permeability, decreased membrane stability, and reduced photosynthetic yield (El-Sheekh et al., 2016; Hamouda et al., 2022; Kocira et al., 2019; Kosakivska et al., 2021). The high levels of phenol and alkaloids can promote the excessive production of free radicals/ROS, which in turn causes damage to cells and DNA (Hamouda et al., 2022; Jesus et al., 2016; Negritto et al., 2017).

The quantification of the relative biomass of FOC in onion root tissues one month after the inoculation of FOC was also accessed in the present study. Results showed that the relative biomass of FOC was significantly higher in ANE treatment, and the relative biomass of FOC in *E. cloacae* CAL2 treatment and the combination treatment were slightly higher than the control group, but no significant difference (Figure 25). The pathogen quantification results were consistent with a reduced fresh (shoot and root) weight in the ANE treatment and a similar fresh (shoot and root) weight in the *E. cloacae* CAL2 treatment, the combination treatment, and the control group (Figure 26). Therefore, these results suggested that the three treatments (ANE, *E. cloacae* CAL2, and their combination) did not induce biochemical changes in onion plants, improve the growth of onion plants, or reduce the pathogen development in onion tissues. Meanwhile, as mentioned before, the single application of a high concentration of ANE may have a certain negative impact on the growth of onion plants, which may make it easier for the pathogen to invade and/or colonize plant tissues. Similarly, a combination of 500 ppm cassia oil and 1% NaCl or KCl was found to control *A. alternata* on tomato plants better than the single treatment of cassia oil, NaCl or KCl. Whereas when higher concentrations (3%) of Kcl or NaCl were used, the combined application did not appear to have a stronger disease control effect than the single treatment (Feng & Zheng, 2006). It is suggested that the application of KCl or NaCl at a high concentration (3%) may damage tomato cells, leading to decreased plant resistance to pathogens (Feng & Zheng, 2006). In contrast with our results, Madriz-Ordeñana et al. (2022) found that the *Bacillus cereus* Strain EC9 significantly reduced the relative amount of *Fusarium oxysporum* in the stems of Kalanchoe (*Kalanchoe blossfeldiana* Poelln.). Some tropical seaweed extracts have been reported to lower DNA concentrations of fungal

and bacterial pathogens in plant tissues as well as increased defence enzyme activities and expression levels of marker genes involved in defence signaling pathways (Ali et al., 2021a; Ramkissoon et al., 2017). In addition, it has been proved that the quantification of fungal DNA can accurately measure the disease severity of *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* (Abdullah et al., 2018; Oliver et al., 2008; See et al., 2016).

In addition, it was interesting that the single application of ANE caused the lowest onion biomass and highest FOC biomass in onion root tissues, but the combination treatment did not, which caused similar onion biomass and FOC biomass as the single application of *E. cloacae* CAL2 and control (Figure 25). This may indicate that the addition of *E. cloacae* CAL2 may alleviate the toxic effect of a high concentration of ANE on onion plants. Several studies have reported that *Enterobacter* strains are able to improve plant growth under stress conditions (Badawy et al., 2022; Bhise et al., 2017; Singh et al., 2017, 2022). For instance, the inoculation of *E. cloacae* ZNP-4 resulted in the alleviation of salinity and metal stress in wheat plants in terms of enhanced plant growth (shoot and root length), biomass production (fresh and dry weights), photosynthetic pigments (chlorophyll a and b), and proline content, and a decreased malondialdehyde level in the presence of NaCl (150 and 200 mM) and $ZnSO_4$ (150 and 250 mg kg^{-1}).

As mentioned above, the two isolated FOC strains from naturally infected onion were identified as *F. oxysporum*, but it remains uncertain whether the two FOC isolates are *F. oxysporum* f. sp. *cepae* or not. The two FOC isolates caused disease symptoms in mature onion bulbs and onion seedlings in the pathogenicity experiments, indicating the pathogenetic capacity of these two FOC isolates. However, pathogen inoculated onion plants showed no disease symptoms in the greenhouse pathogenicity test and greenhouse

experiment. Therefore, the disease incidence and disease severity were unable to be accessed as disease symptoms were not consistently reproduced, and the developed disease symptoms were too mild and not distinct enough. However, during field sampling, patches of diseased onion plants were found in the field, with symptoms of yellowing, wilted and curled leaves, and basal/bulb rot (Figure 27). There are several factors involved in the failure of pathogen-inoculated onion plants to develop disease symptoms under greenhouse condition. Greenhouse experiment and field experiment differ in several biotic and abiotic aspects that might be the reason behind the differences in the development of disease symptoms. The intensity of disease progression could be influenced by many factors, such as environmental factors, virulence and aggressiveness of the pathogen(s), and biotic interactions between microorganisms, macroorganisms, and pathogens (Borer et al., 2016; Yurgel et al., 2018). Environmental factors like water, temperature and soil type, and biotic factors like soil microorganisms and macroorganisms are different in field conditions than in greenhouse conditions with a strongly controlled environment. For instance, onion thrips can induce physical damage to the plants which may provide additional entrances that facilitate the invasion or infection of fungal pathogens (Alyokhin et al., 2020). Drought stress has been reported to exacerbate fungal colonization and increase the incidence and severity of dry root rot disease in chickpea (Irulappan et al., 2022). Liu and Liu (2016) found that high soil moisture facilitates the initial stage of *Fusarium pseudograminearum* infection, and drought stress increases Fusarium pathogen proliferation and spread after the initial phase of infection. Previous studies have also mentioned that soil temperature was associated with the appearance of disease, and a higher soil temperature was associated with a higher disease incidence (Abawi, 1972; Kehr et al., 1962; Szatala, 1969; Walker &

Tims, 1924). It was reported that the infection of FOC in onion plants occurred first in early July when the temperature was over 25℃ (Kehr et al., 1962). Haapalainen et al. (2016) found that the virulence of *F. oxysporum* isolates to onion varied greatly. They also indicated that the time needed for different isolates develop on onion may vary, which may affect the time needed for symptoms development. It was also reported that the frequent subculture of fungal isolates on carbohydrate-rich media might result in the decrease of virulence of the isolates, which is commonly seen in *F. oxysporum* species (Leslie & Summerell, 2006; Paynter et al., 2016).

Figure 27. Onion plants with symptoms of yellowing, wilted and curled leaves in the field (A). Collected diseased onion bulbs from the field showed symptoms of basal rot when cutting open (B).

CHAPTER 6 : CONCLUSION

Many studies have been conducted on onion Fusarium basal rot management using different methods, such as commercial fungicide treatments, biocontrol management, and breeding resistant cultivars. There is no research that has been performed so far about using seaweed extract or the combination of seaweed extract and PGPR to control onion Fusarium basal rot disease.

The present study provides useful information on the biocontrol efficiency of three PGPR strains (*Pseudomonas. protegens* CHA0, *Enterobacter cloacae* CAL2, and *Bacillus subtilis*), *A. nodosum* extract (ANE), and their combination against Fusarium basal rot disease caused by *Fusarium oxysporum* f.sp. *cepae* (FOC) in onion under *in vitro* and *in vivo* conditions. The *in vitro* tests showed that three PGPR strains and each combined with ANE successfully suppressed the FOC. It was also observed that *E. cloacae* CAL2 and the combination of *E. cloacae* CAL2 and ANE gave the best inhibition effect of mycelial growth and spore germination of the FOC in comparison with other PGPR strains alone or combined with ANE. Although a certain concentration of ANE enhanced the growth of some PGPR strains, ANE itself did not show any antifungal activity nor enhance the antifungal effect of the tested PGPR strains.

Conversely, the *in vivo* results underline the application of *E. cloacae* CAL2, and ANE, and their combination did not significantly improve the growth of onion plants. Meanwhile, the single treatment of ANE caused a reduction in shoot and root biomass of onion and an increase in pathogen biomass in root tissue, which could be due to the toxicity effect of the

high salinity or the high levels of minerals and antioxidants (e.g., phenols and alkaloids) in ANE.

Overall, the results of the present study could potentially contribute to the development of the combination of biocontrol agents for the management of onion FBR disease and other plant diseases. Further studies are needed to investigate the combinations of different PGPR strains and seaweed extract at different concentrations under both *in vitro* and *in vivo* conditions in order to find the most effective combination, and to understand how they function in plant defence response pathways, and investigate their full potential for controlling plant diseases.

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APPENDIX A SEEDLING PATHOGENICITY TEST

A.1 METHODOLOGY

The pathogenicity tests of each FOC isolate were also performed on healthy onion seedlings according to the method described by Degani and Kalman (2021). The onion (Utah Sweet Spanish) seeds were sterilized by soaking in 1% NaOCl for 3 min and then rinsed thrice in SDW.

A.1.1 MYCELIAL DISC INOCULATION

Sterilized onion seeds were transferred to a Petri dish in which a sterile Whatman paper (Each Petri dish with 15 seeds) was soaked in SDW (6 ml). Seeds were inoculated by placing a mycelial disc (6 mm), which was cut from the growing edge of a seven-day-old culture of FOC, on the center of each Whatman paper in each Petri dish. A sterile PDA disc (6 mm) was placed in the control. Sterilized distilled water was added to each plate every three days to maintain moisture and to allow efficient germination and disease progression. The plates were incubated at 28±2°C in the dark for 9 days. The seeds were photographed, and the germination percentage was measured. The experiment was repeated three times with four replicates.

A.1.2 SPORE-CMC COATING INOCULATION

Sterilized onion seeds were soaked in FOC spore-suspension (1% CMC, 10^6 spores/ml) for 30 min so that the seeds were properly coated with spores and then air dried in a laminar flow hood. Inoculated seeds were transferred to a Petri dish in which a sterile Whatman paper (Each Petri dish with 15 seeds) was soaked in SDW (6 ml). Sterilized onion seeds soaked in 1% CMC solution served as the control. Sterilized distilled water was added to

each plate every three days to maintain moisture and to allow efficient germination and disease progression. The plates were incubated at 28±2°C in the dark for 9 days. The seeds were photographed, and the germination percentage was measured. The experiment was repeated three times with four replicates.

A.2 RESULTS

For the mycelial disc inoculation method, according to Figure A1, there were white mycelial on or near onion seeds in the Fusarium-inoculated groups. Meanwhile, the Fusarium-inoculated groups had a lower seed germination rate than the control group (Figure A2). The same symptoms were also absorbed in the spore-CMC coating inoculation method (Figures A3&A5). In addition, the Fusarium-inoculated groups had lower shoot lengths than the control group in the spore-CMC coating inoculation method (Figures A4&A5).

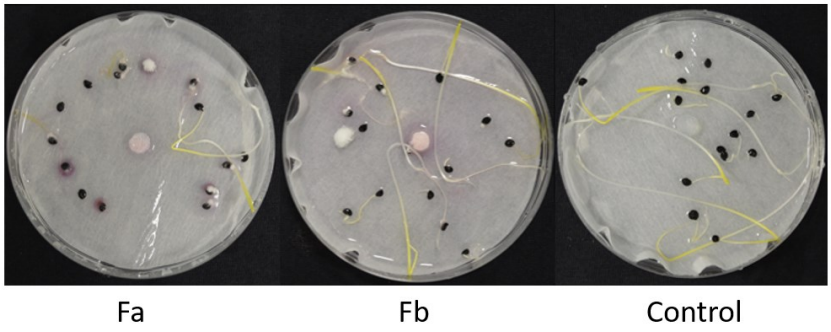

Figure A 1. Image of the seedling pathogenicity test by using the mycelial disc inoculation method. Onion seeds were inoculated with Fa and Fb (two FOC isolates) and Control (Clear PDA agar plug).

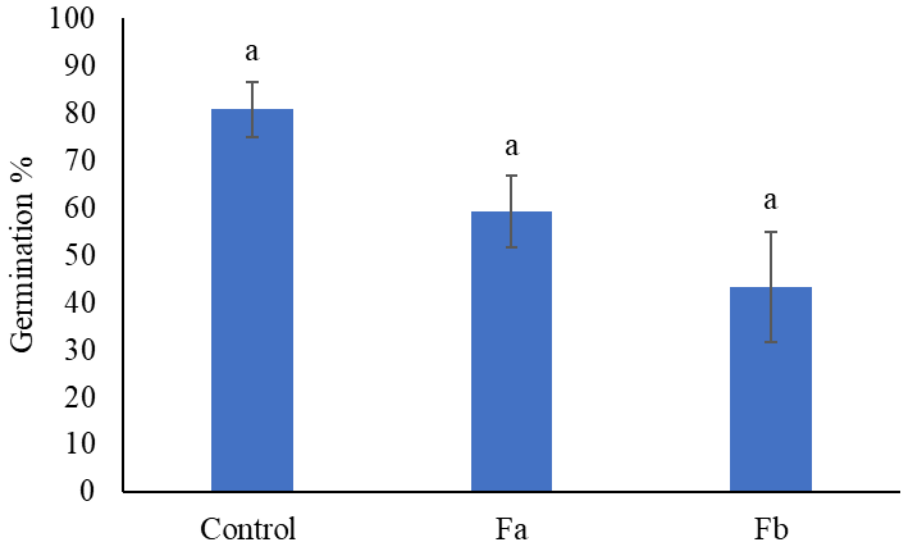

Figure A 2. The germination rate of onion seeds inoculation with two FOC isolates (Fa and Fb) and Control (Clear PDA agar plug) by using the mycelial disc inoculation method. Data were analyzed using One-Way ANOVA. Error bars represent SE. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

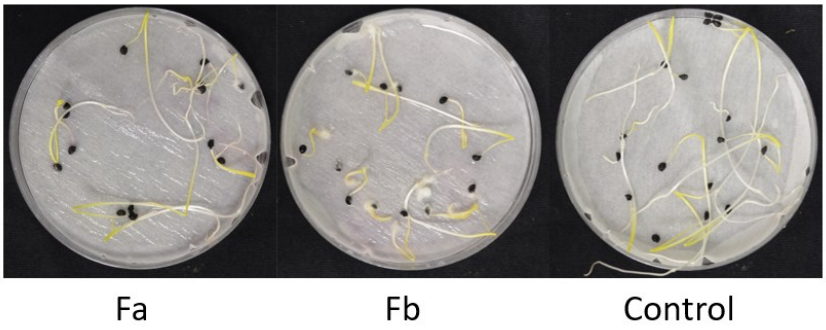

Figure A 3. Image of the seedling pathogenicity test by using the spore-CMC coating inoculation method. Onion seeds were inoculated with two FOC isolates (Fa and Fb) and Control (1%CMC).

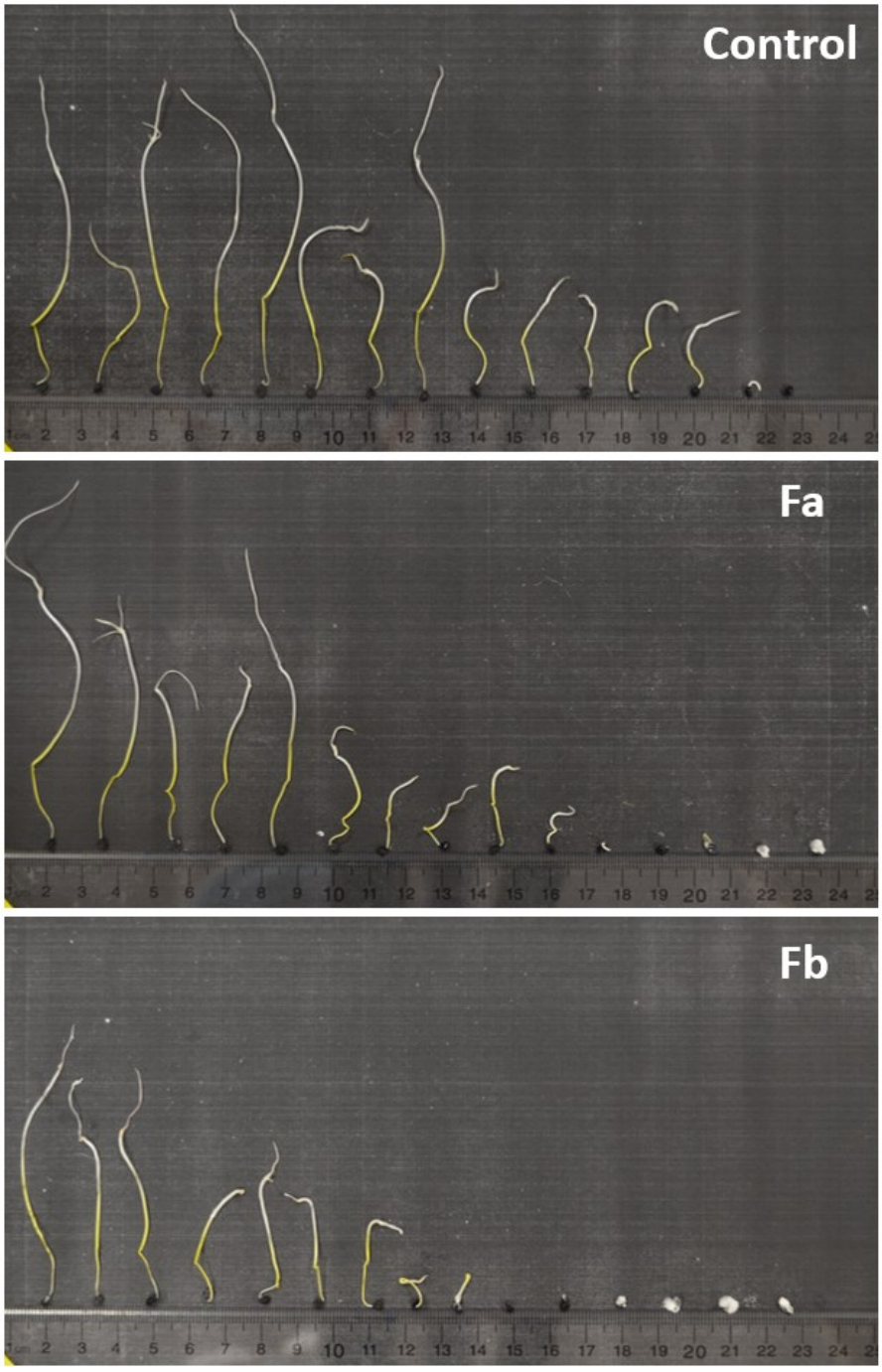

Figure A 4. Image of the shoot length of onion seedlings in seedling pathogenicity test. Onion seeds were inoculated with two FOC isolates (Fa and Fb) and Control (1%CMC) by using the spore-CMC coating inoculation method.

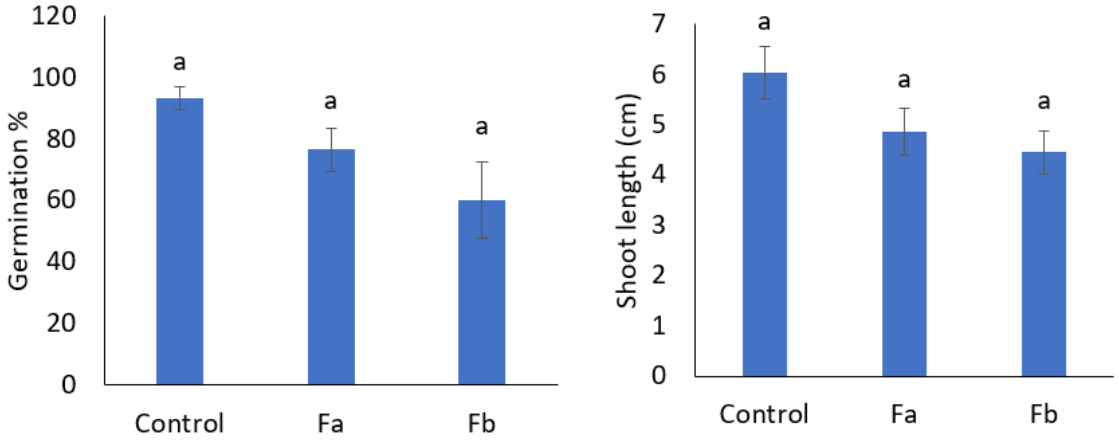

Figure A 5. The germination rate (left) and average shoot length (right) of onion seeds inoculation with two FOC isolates (Fa and Fb) and Control (Clear PDA agar plug) by using spore-CMC coating inoculation method. Data were analyzed using One-Way ANOVA. Error bars represent SE. Bars with different letters are significantly different ($P \leq 0.05$; Tukey's multiple comparisons).

APPENDIX B POT PATHOGENICITY TEST

B.1 METHODOLOGY

B.1.1 ROOT DIPPING AND TOPSOIL INOCULATION METHODS

Onion sets (Yellow Dutch) were sterilized by soaking in 1% NaOCl for 3 min and then rinsed thrice in distilled water. Then, onion sets were planted in Pro-mix BX (without mycorrhizae), and then placed in a growth chamber, which was adjusted to 22±2°C (day/night) with a photoperiod of 16 h (cool white fluorescent tube lights, 150 μmol m^{-2} s^{-1}). One month after germination, onion seedlings were carefully removed from Pro-mix, and the roots were washed. For the root dipping group, the roots of onion seedlings were cut to 6 cm and then inoculated with FOC spore suspension (10^5 spores/ml) by root dipping method for 30 min. The control group was dipped the onion roots in SDW. Then, onion plants were transplanted into pots filled with Pro-mix at a rate of four plants per pot. For the topsoil inoculation group, each pot was first filled with 200g Pro-mix, then another 150g Pro-mix, which was inoculated with FOC spore suspension (10^5 spores/ml), was added on top of the previously added Pro-mix. Then, after the roots were washed, onion seedlings were transplanted into each pot at a rate of four plants per pot. For the control group, 150g Pro-mix was mixed with the same amount of SDW as FOC spore suspension.

For two groups, there were 24 pots per group, 12 pots were inoculated with control, and another 12 pots were the control. As shown in Figure B1, the Pro-mix in half of the inoculated or control pots was kept normal moist, and another half of the pots were kept drought by adding tap water as needed.

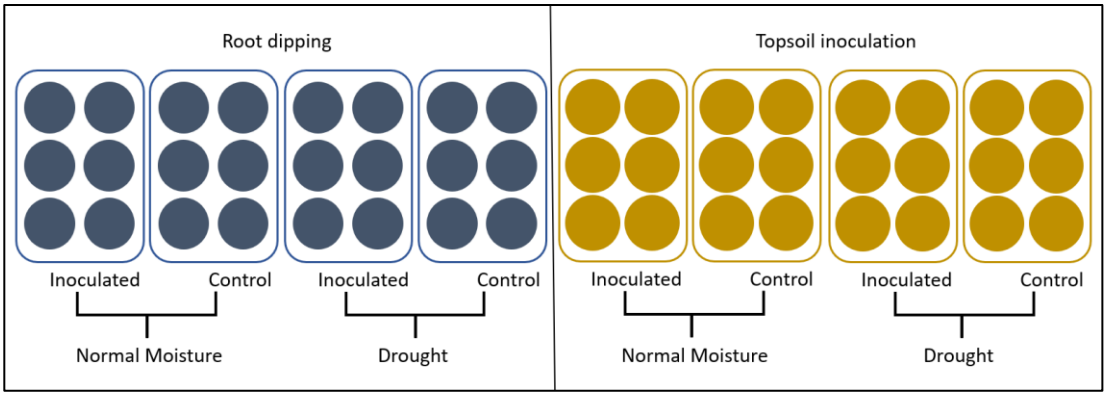

Figure B 1. A layout of root dipping and top-soil inoculation pot pathogenicity test.

B.1.2 DIFFERENT TYPES OF SOIL MATRIX

Filed soil was collected from Dalhousie Agricultural Campus Demonstration Garden (NS, Canada) and sterilized by using an autoclave at 15psi and 121°C for 30 min on two consecutive days. Soil type 1 was sterilized field soil mixed with sand and Pro-mix in a ratio of 4:1:1 (w/w/w), and soil type 2 was sterilized field soil mixed with perlite in a ratio of 25:1 (w/w/w).

One-month-old onion seedlings were carefully removed from Pro-mix, and the roots were washed. The roots of onion seedlings were cut to 6 cm and then inoculated with FOC spore suspension (Fa and Fb, 10^5 spores/ml) by root dipping method for 30 min. The control group was dipped the onion roots in SDW. Then, onion plants were transplanted into pots filled with each type of soil mixture (1000 g) at a rate of three plants per pot. For two groups, as shown in Figure B2, there were 18 pots per group.

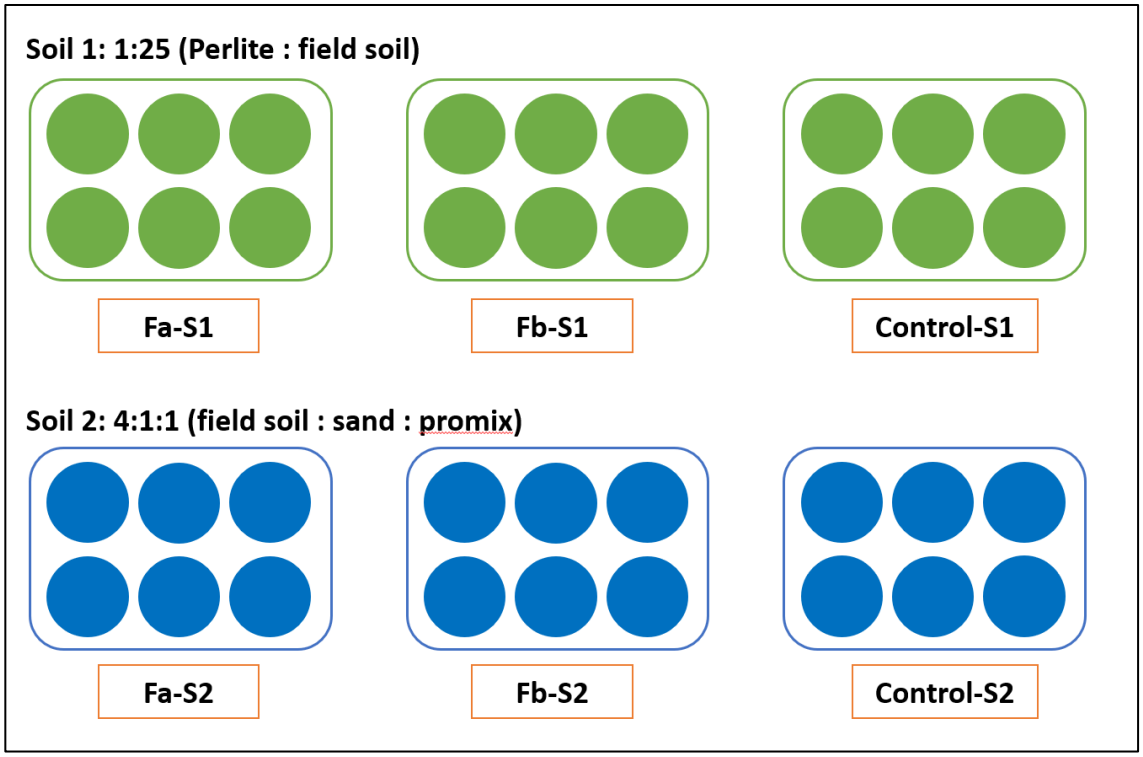

Figure B 2. A layout of pot pathogenicity test by using different soil matrix.

B.2 RESULTS

For root dipping and topsoil inoculation methods, onion seedlings in root dipping groups developed symptoms of transplant chock such as plant flattened and wilting leaves (Figure B3). One month after inoculation, we only observed that a few roots turned brown to dusky pink and the roots of inoculated onion in the root dipping group were very easily lost when we removed them from the Pro-mix. The above-ground parts of the inoculated onions in two inoculation methods did not show any symptoms of Fusarium basal rot disease as expected (Figure B5).

For different soil matrix methods, we also observed that the onion plants showed symptoms of transplant shock, and plants died a few days after transplantation, especially in soil type 1, in which all the plants gradually died within two weeks after transplantation. Transplant

shock can be caused by cut roots and the field soil was too compressed. For soil type 2, the roots of onion plants were also very easily lost when we removed them from the soil as the soil was too compressive. However, the above-ground parts of the inoculated onions in the two methods also did not show any symptoms of Fusarium basal rot disease as expected (Figure B6). There were some plants died, but it was uncertain whether it was caused by disease or transplant shock.

Figure B 3. Onion seedling wilt after transplanting.

Figure B 4. Roots of inoculated onion plants turn brown to dusky pink.

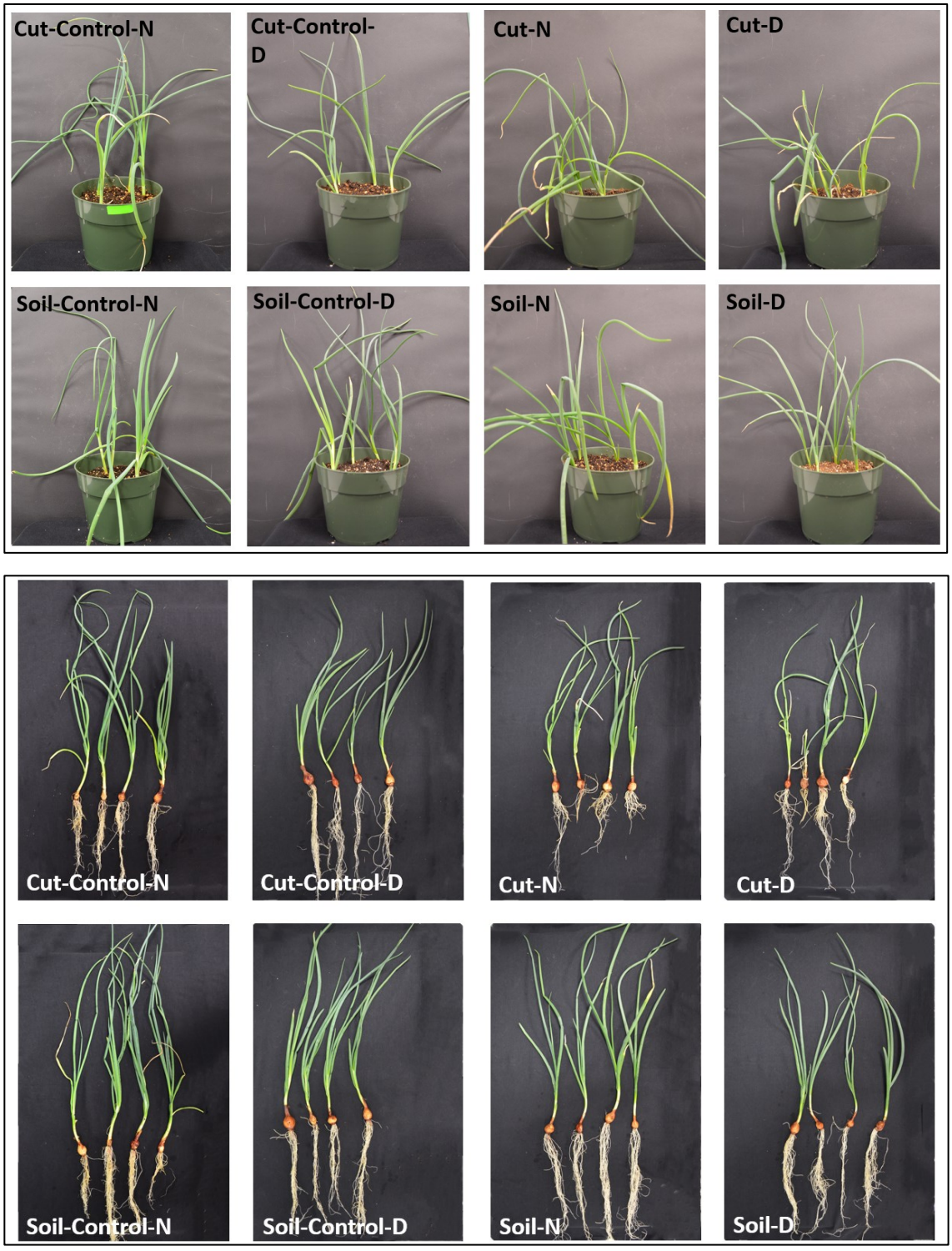

Figure B 5. Image of onion plants (plant in pots and uprooted plants.) 30 days post inoculation in pot pathogenicity test by using root dipping and topsoil inoculation methods.

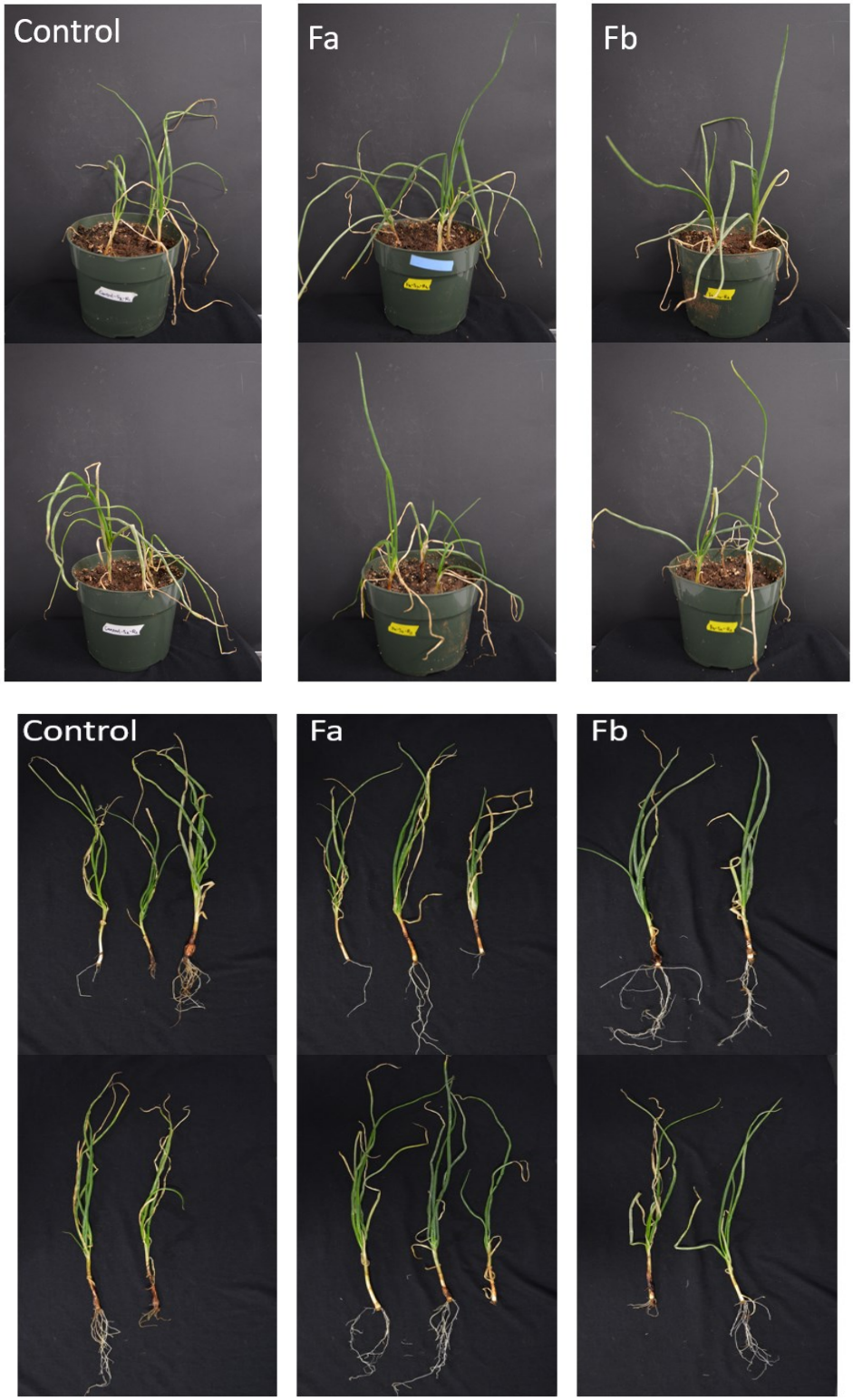

Figure B 6. Image of onion plants (Plants in pot and uprooted plants) 30 days post inoculation in pot pathogenicity test by using sterilized field soil, sand, and Pro-mix in a ratio of 4:1:1 (w/w/w).

APPENDIX C DUAL CULTURE ASSAYS

C.1 METHODOLOGY

Dual culture assay is the most common technique used to test the antagonistic activity of a selected bacterial isolate against the target fungal phytopathogen. In this project, we tried different modified dual culture assays to test the effect of *P. protegens* CHA0 and/or ANE on the mycelial growth of FOC. Then, use the appropriate method to screen other PGPR strains against FOC.

P. protegens CHA0 and ANE were tested individually and in combination against FOC by using dual culture bioassays with some modifications (Dennis & Webster, 1971). Agar plugs of *P. protegens* CHA0 and FOC were cut from the growing edge of seven-day-old cultures of *P. protegens* CHA0 and FOC, respectively. ANE powder was dissolved in water and added at a concentration of 0, 0.1, and 0.2% (w/v) to the PDA medium before being autoclaved and cooled.

C.1.1 AGAR PLUG - PDA MEDIUM

The experiment was set up as follows:

For *P. protegens* CHA0 alone: An agar plug (5 mm) colonized by FOC and an agar plug (5 mm) colonized by *P. protegens* CHA0 were placed 2 cm from the edge of the PDA plate respectively opposite to each other.

For ANE alone: The PDA plate was amended with different concentrations of ANE (0.1% and 0.2%). An agar plug (5 mm) colonized by FOC and a clear agar plug (5 mm) were placed 2 cm from the edge of the PDA plate, respectively opposite to each other.

For *P. protegens* CHA0 and ANE combination: The PDA plate was amended with different concentrations of ANE (0.1% and 0.2%). An agar plug (5 mm) colonized by FOC and an agar plug (5 mm) colonized by *P. protegens* CHA0 were placed 2 cm from the edge of the PDA plate, respectively, opposite to each other.

For control: An agar plug (5 mm) colonized by FOC and a clear agar plug (5 mm) were placed 2 cm from opposite edges of the PDA plate.

The experiment was repeated three times with three replicates.

C.1.2 STREAKING - PDA

The experiment was set up as follows:

For *P. protegens* CHA0 alone: An agar plug (5 mm) colonized by FOC was placed 2 cm from the edge of a PDA plate. A single colony of the *P. protegens* CHA0 from a seven-day-old culture of *P. protegens* CHA0 was streaked in a straight line (7 cm) 4 cm away from the agar plug of FOC in the PDA plate.

For ANE alone: PDA plate was amended with different concentrations of ANE (0.1% and 0.2%). An agar plug (5 mm) colonized by FOC was placed 2 cm from the edge of the PDA plate.

For *P. protegens* CHA0 and ANE combination: PDA plate was amended with different concentrations of ANE (0.1% and 0.2%). An agar plug (5 mm) colonized by FOC was placed 2 cm from the edge of a PDA plate. A single colony of the *P. protegens* CHA0 from a seven-day-old culture of *P. protegens* CHA0 was streaked in a straight line (7 cm) 4 cm away from the agar plug of FOC in the PDA plate.

For control: An agar plug (5 mm) colonized by FOC was placed 2 cm from opposite edges of a PDA plate.

The experiment had three replicates.

C.1.3 STREAKING - PDA, HALF LB, AND HALF KB MEDIUM

P. protegens CHA0 was cultured into the liquid broth and incubated overnight at 28±2˚C, 180 rpm. The overnight culture of *P. protegens* CHA0 was diluted to 1:100 with fresh broth and incubated at 28±2˚C, 180 rpm till the OD_{600} reached 0.6, which was used as stock culture. The stock culture was diluted to 1:100 with fresh broth, which was supplemented with different concentrations of ANE (0% and 0.1%). After 10 h incubation (28±2˚C, 180 rpm), 10 µl of *P. protegens* CHA0 culture was streaked in a straight line (5 cm) 2 cm away from the edge of a new PDA/half LB/half KB plate one day before the FOC inoculation.

An agar plug (5 mm) of FOC was cut from the growing edge of a seven-day-old culture of FOC and inoculated 4 cm away from the bacterial inoculation in the edge of the PDA/half LB/half KB plate. Plates inoculated with the fungal disc and clean liquid broth were used as control. The experiment had three replicates.

C.1.4 SCREENING PLANT GROWTH PROMOTING RHIZOBACTERIA AGAINST FOC

The method used in this part was mentioned in part A.1.3, and the experiment was done on PDA and different bacteria media. The plant growth promoting rhizobacteria tested in this part are in Table C1. The experiment had two replicates.

For these experiments, all plates were placed into the incubator (28±2°C) for 7 days, and then the inhibition zone was measured. The inhibition percentage was calculated by using the equation (Muniroh et al., 2019):

$$\text{Inhibition percentage (\%)} = \frac{R_c - R_t}{R_c} \times 100\%$$

Where:

R_c was the radial growth of the fungal colony in the control plate

R_t was the radial growth of the fungal colony in the presence of PGPR and/or ANE

Table C 1. List of PGPR and their respective culture media.

No.	Name	Medium
19	*Serratia proteamaculans* subsp. Quinovara	Tryptic soy agar (TSA)
31	*Enterobacter agglomerans* ATCC23216	Nutrient agar (NA)
37	*Pseudomonas putida* GR12-2	TSA
54	*Pseudomonas fluorescens* 34-13	TSA
89	*Enterobacter cloacae* CAL2	TSA
163	*Azotobacter vinelandii* ATCC 12837	NA
181	*Azotobacter vinelandii*	NA
213	*Azospirillum lipoferum* 1842	NA
232	*Pseudomonas brassicacearum* Zy-2-1	Yeast Mannitol Agar (YMA)
279	*Bacillus thuringiensis* subsp. oloke	PDA
303	*Kluyvera ascorbata* SUD165	NA
431	*Sinorhizobium fredii* ATCC51808	YMA
532C	*Bradyrhizobium japonicum*	YMA
535	*Paenibacillus polymyxa* P.p K56	LB
562	*Burkholderia cepacia* IS-16	NA
612	*Sinorhizobium meliloti* 11559	LB
613	*Sinorhizobium meliloti* 8530	LB
744	*Mesorhizobium* strain PM-14	YMA

C.2 RESULTS

The results of Part C.1.1. showed that the treatment of *P. protegens* CHA0 and the combination treatment of *P. protegens* CHA0 and ANE (0.1% and 0.2%) significantly

inhibited the mycelial growth of FOC, but the inhibition percentage was only between 9% to 16% (Table C2 & Figure C1). The ANE treatments did not exhibit significant pathogen inhibition. We also observed that the bacteria grew very slowly as we used the agar plug method to inoculate it to the plate, which could result in the bacteria cannot reach their full potential.

The results of Part C.1.2. showed that only the treatment of *P. protegens* CHA0 significantly inhibited the mycelial growth of FOC. The addition of ANE to the PDA media may not be an appropriate method because the bacteria may not be able to fully absorb or use the ANE in the media. In addition, the type of culture medium may also be affected by the antagonistic activity of the bacteria (Table C3&Figure C2).

The results of Part C.1.3 showed that the antagonistic activity of *P. protegens* CHA0 was highest in the PDA media instead of half KB or LB media. Meanwhile, there was no pigment production of FOC in half KB or LB media (Table C4&Figure C3).

The results of Part C.1.4. showed that the 89 (*E. cloacae* CAL2) exhibited the highest mycelial growth inhibition percentage in PDA media compared with other bacteria in PDA media or bacteria media. It was found again that there was no pigment production of FOC in bacteria media (Tables C5-6&Figures C4-5).

Table C 2. Dual culture assay of *P. protegens* CHA0 and/or ANE (0.1% and 0.2%) against the pathogen mycelial growth. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

Treatment	Inhibition Percentage (%)±SE	
CHA0	16.2±2.9	a
0.1%ANE+CHA0	11.8±3.7	ab
0.2%ANE+CHA0	9.3±4.0	ab
0.1%ANE	5.1±2.5	bc
0.2%ANE	1.7±2.2	c
Control	0	c

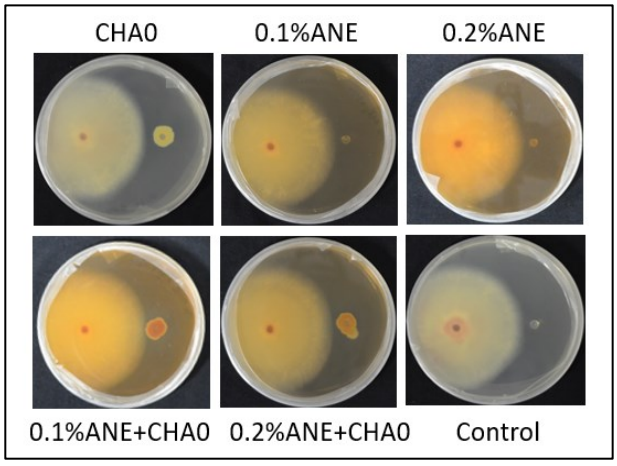

Figure C 1. Suppression of mycelial growth of FOC by *P. protegens* CHA0 and/or ANE (0.1% and 0.2%) under *in vitro* conditions by using a dual culture test.

Table C 3. Dual culture assay of *P. protegens* CHA0 and/or ANE (0.1% and 0.2%) against the pathogen mycelial growth. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

Treatment	Inhibition Percentage (%)±SE	
CHA0	13.3±2.3	a
0.2%ANE+CHA0	9.9±2.8	ab
0.1%ANE+CHA0	8.3±0.9	ab
0.2%ANE	7.4±2.9	ab
0.1%ANE	4.2±3.0	ab
Control	0.0	b

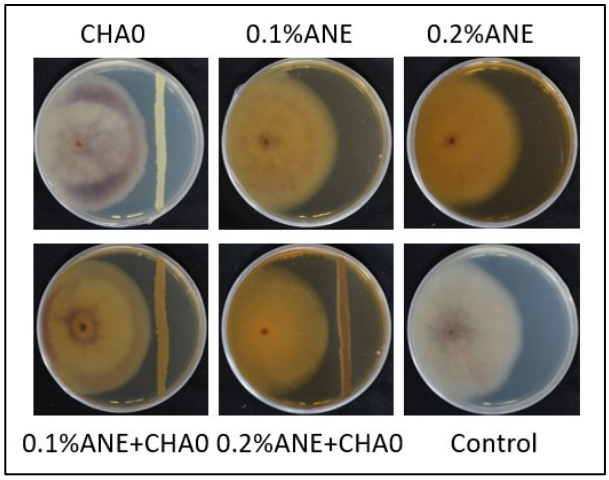

Figure C 2. Suppression of mycelial growth of FOC by *P. protegens* CHA0 and/or ANE (0.1% and 0.2%) under *in vitro* conditions by using a dual culture test.

Table C 4. Dual culture assay of *P. protegens* CHA0 and the combination of *P. protegens* CHA0 and ANE (0.1%) against the pathogen mycelial growth in different culture media.

Treatment	Inhibition Percentage±SE		
	PDA	Half LB	Half KB
CHA0	19.2±2.4	4.2±2.2	0.1±3.1
CHA0+0.1%ANE	15.5±0.0	4.2±6.6	2.2±0.7
Control	0.0	0.0	0.0

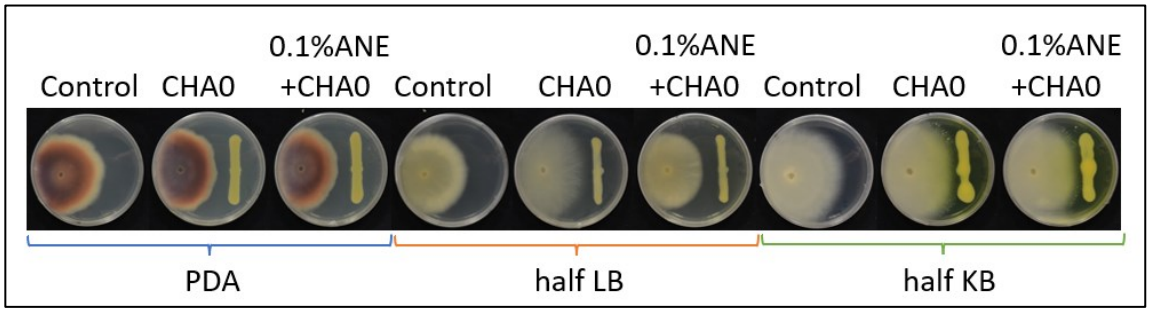

Figure C 3. Suppression of mycelial growth of FOC by *P. protegens* CHA0 and the combination of *P. protegens* CHA0 and ANE (0.1%) in different culture media under *in vitro* conditions by using a dual culture test.

Table C 5. Dual culture assay of different PGPR strains against the pathogen mycelial growth in PDA media. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

	PDA	
Treatment	**Inhibition Percentage±SE**	
89	43.9±0.0	a
612	24.4±2.4	b
279	22.0±0.0	bc
37	19.5±0.0	bc
562	18.3±1.2	bc
431	17.1±2.4	bcd
744	15.9±1.2	bcde
535	15.9±3.7	bcde
613	14.6±2.4	bcdef
232	13.4±1.2	bcdef
31	13.4±3.7	bcdef
19	12.2±0.0	cdef
54	11.0±3.7	cdefg
303	11.0±1.2	cdefg
181	6.1±1.2	defg
532C	4.9±2.4	efg
163	4.9±2.4	efg
213	3.7±1.2	fg
C	0.0	g

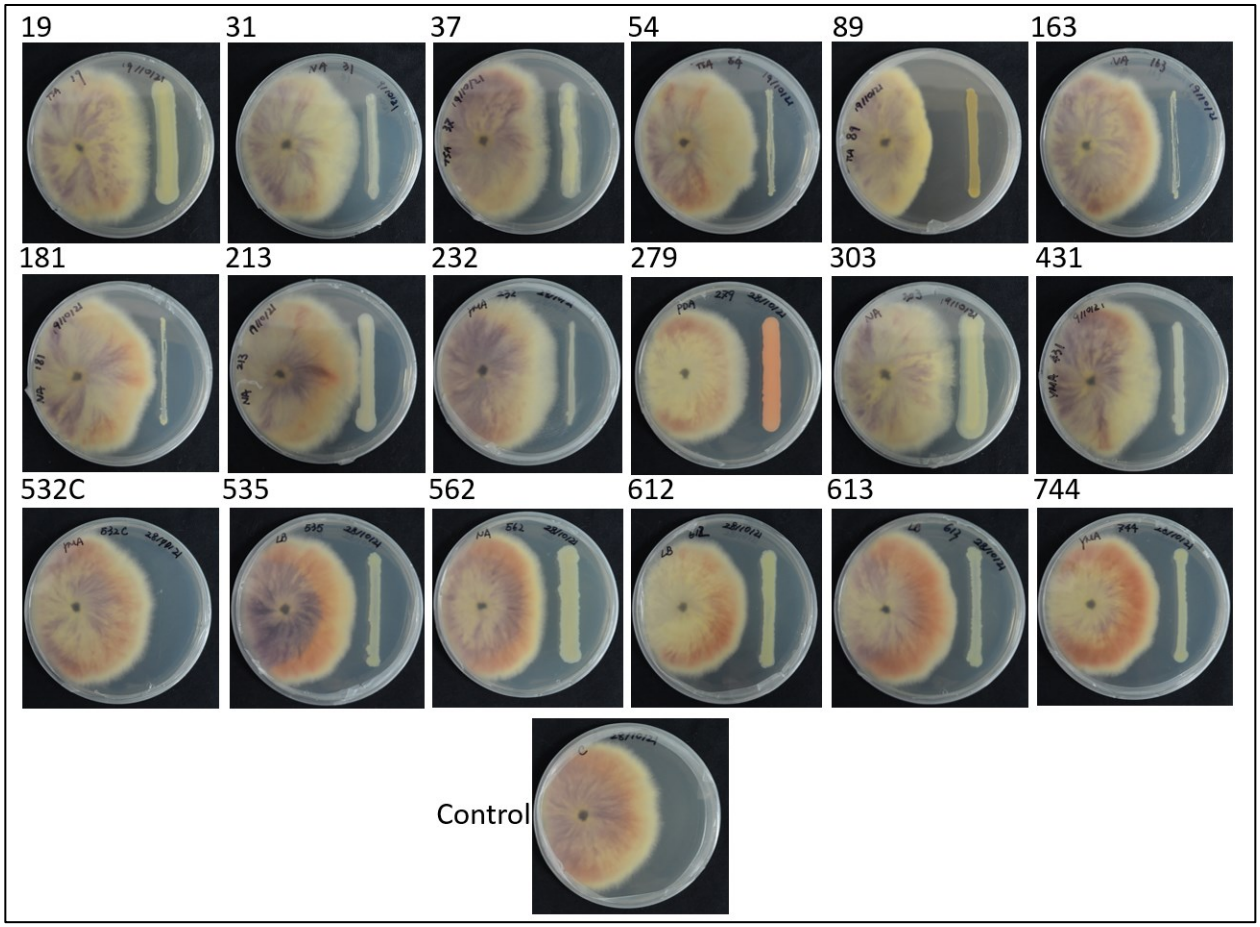

Figure C 4. Suppression of mycelial growth of FOC by different PGPR strains in PDA media under *in vitro* conditions by using a dual culture test.

Table C 6. Dual culture assay of different PGPR strains against the pathogen mycelial growth in different bacteria culture media. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

Treatment	Inhibition Percentage±SE	
	YMA	
744	21.8±0.0	a
431	13.5±0.0	b
532c	5.5±3.6	c
232	4.6±0.9	c
C	0.0	c
	TSA	
19	38.4±3.5	a
54	30.2±0.0	a
89	19.8±5.8	ab
37	14.0±9.3	ab
C	0.0	b
	NA	
562	33.0±1.1	a
303	27.3±2.6	ab
163	23.4±1.3	ab
181	22.1±5.2	ab
213	20.8±1.3	ab
31	9.1±10.4	bc
C	0.0	c
	LB	
612	32.3±1.0	a
613	28.3±1.0	a
535	22.2±1.0	b
C	0.0	c
	PDA	
279	20.2±3.6	a
C	0.0	b

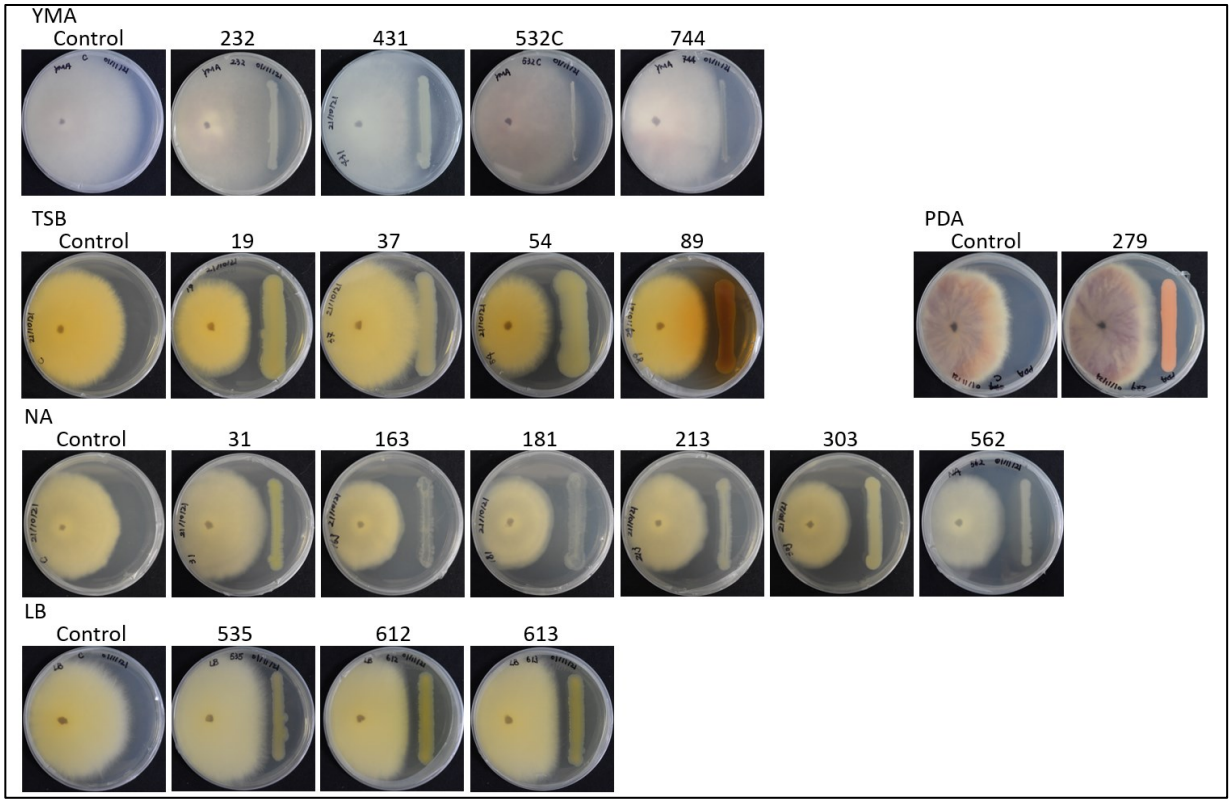

Figure C 5. Suppression of mycelial growth of FOC by different PGPR strains in different bacteria culture media under *in vitro* conditions by using a dual culture test.

APPENDIX D SPORE GERMINATION ASSAY

D.1 METHODOLOGY

In this project, we tried different modified hanging drop methods to test the effect of PGPR and/or ANE on the spore germination of FOC (Fang, 1998).

D.1.1 *P. PROTEGENS* CHA0 GROW ON AGAR PLATE

Colonies of *P. protegens* CHA0 were harvested from two-day-old cultures of *P. protegens* CHA0 by adding 5 ml of SDW in the Petri plate and gentle scrubbing. Uniform cell density was maintained at 10^7 c.f.u./ml. The FOC spore suspension (40 µl, 10^5 spores/mL) was mixed with 40 µl of different treatments, which were *P. protegens* CHA0 suspension (10^7 c.f.u./ml), 0.1% ANE, 0.1% ANE with *P. protegens* suspension (10^7 c.f.u./mL). The mixture of FOC spore suspension (40 µl) and distilled water (40 µl) was used as a control. The total volume of the mixed suspension was 80 µl. The mixed suspension was dropped on a piece of the concave slide. The slides were incubated for 6h at 28±2˚C in a moist condition.

D.1.2 *P. PROTEGENS* CHA0 GROW ON LIQUID CULTURE

A stock culture of CHA0 cells (0.6 OD600) was prepared as described in section C.1.3. The stock culture of *P. protegens* CHA0 ($OD_{600=0.6}$) was diluted to 1:100 with LB/KB broth, which has been supplemented with different concentrations of ANE (0% and 0.1%). After 10h incubation (28±2˚C, 180 rpm), living cells of *P. protegens* CHA0 were harvested by centrifugation. The resulting supernatant was then filtered through a syringe filter (VWR® syringe filters, PTFE, 0.2µm) to prevent the presence of bacterial cells. The treatments were CHA0 living cells (CHA0-L), CHA0 living cells+0.1%ANE (CHA0-

L+ANE), CHA0 supernatant (CHA0-S), 0.1%ANE+CHA0 supernatant (ANE+CHA0-S), 0.1%ANE, and 0.1%ANE-LB/KB broth. The FOC spore suspension (40 µl, 10^5 spores/mL) was mixed with each treatment. The mixture of FOC spore suspension (40 µl) and distilled water (40 µl) or LB/KB broth (40 µl) were used as control groups. The total volume of the mixed suspension was 80 µl. The mixed suspension was dropped on a piece of the concave slide. The slides were incubated for 6h at 28±2˚C in a moist condition.

For all experiments, spore morphology was observed in five different views under a compound light microscope. A total of 100 spores were randomly counted and recorded in each view. The number of germinated spores among those spores in each view was counted and recorded. Germination was considered when the length of the germ tube reached half the size of the spore. Each experiment was replicated three times with 3 replicates. The germination rate was calculated as follow:

$$\text{Germination rate (\%)} = \frac{\text{Total counted germinated spores in five views}}{\text{Total counted spores in five views}} \times 100\%$$

D.2 RESULTS

The results of Part D.1.1 showed that the treatment of *P. protegens* CHA0, and the combination treatment of *P. protegens* CHA0 and 0.1%ANE significantly inhibited the spore germination of FOC and the ANE treatments did not inhibit germination of FOC spores. We observed that the spore germination rate in control was only around 57% (Table D1). Therefore, we extend the incubation hour from 4h to 6h in the following experiments.

The results of Part D.1.2 showed that, compared with the control (water) group, all treatments except 0.1%ANE exhibited an inhibitory effect on spore germination (Table

D2). However, the spore germination rate of the control (LB) was 24%, which was no different from that of the other treatments that containing LB broth (CHA0-L, CHA0-L+0.1%ANE, CHA0-S, 0.1%ANE+CHA0-S, 0.1%ANE-LB). Similar results were also found in the KB broth group (Table D3).

Table D 1. Inhibitory effect of *P. protegens* CHA0 and/or ANE on spore germination of the pathogen. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

Treatment	Germination rate±SE	
Control	57.1±1.8	a
ANE	48.7±4.7	a
CHA0	22.8±6.9	b
CHA0+ANE	23.4±0.1	b

Table D 2. Inhibitory effect of *P. protegens* CHA0 living cells (CHA0-L) or cell-free supernatant (CHA0-S) in LB broth, 0.1%ANE, 0.1%ANE in LB broth and their combination (0.1%ANE+CHA0-S, CHA0-L+0.1%ANE) on spore germination of the pathogen. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

Treatment	Germination rate±SE	
Control (water)	74.9±4.9	a
0.1%ANE	63.5±3.3	a
0.1%ANE-LB	30.5±1.0	b
0.1%ANE+CHA0-S	29.3±6.6	b
Control (LB broth)	23.7±3.3	b
CHA0-S	21.9±3.3	b
CHA0-L	15.8±1.4	b
CHA0-L+0.1%ANE	10.5±0.6	b

Table D 3. Inhibitory effect of *P. protegens* CHA0 living cells (CHA0-L) or cell-free supernatant (CHA0-S) in KB both, 0.1%ANE, 0.1%ANE in KB broth and their combination (0.1%ANE+CHA0-S, CHA0-L+0.1%ANE) on spore germination of the pathogen. Data were analyzed using One-Way ANOVA. Grouping was done using Tukey's pairwise comparison and 95% confidence.

Treatment	Germination rate±SE	
Control (water)	67.5±1.8	a
0.1%ANE	64.6±3.3	a
0.1%ANE+CHA0-S	36.7±0.8	b
CHA0-S	36.3±3.3	b
Control (KB broth)	34.9±1.1	b
0.1%ANE-KB	34.7±2.2	b
CHA0-L	28.6±1.5	bc
CHA0-L+0.1%ANE	19.0±2.4	c

APPENDIX E THE ACTIVITY OF ANTIFUNGAL SUBSTANCES PRODUCED BY *P. PROTEGENS* CHA0 IN MYCELIAL GROWTH OF FOC

E.1 METHODOLOGY

E.1.1 FILTER PAPER DISC DIFFUSION METHOD

The influence of ANE on the inhibitory effect of bacterial secondary metabolites on the mycelial growth of FOC was accessed by the disc diffusion assay (Bayer et al., 1966) and agar diffusion assay (Choyam et al., 2015).

Mycelial disc inoculation

The stock culture of *P. protegens* CHA0 ($OD_{600=0.6}$) was diluted to 1:100 with LB broth, which was supplemented with different concentrations of ANE (0% and 0.1%) and incubated for one to seven days at 28±2°C and 180 rpm. The culture supernatant for each day was collected after centrifugation at 9600 rpm for 15 min. The resulting supernatant was then filtered through a syringe filter (VWR® syringe filters, PTFE, 0.2µm) to prevent the presence of bacterial cells. Clean LB broth was used as the control. A mycelial disc (6 mm) was cut from the growing edge of a seven-day-old culture of FOC and then placed on the center of each new PDA plate. The filtered supernatants (15 µl) were spotted onto each sterilized filter paper disc (6 mm), which had been placed on each PDA plate 3cm away from each mycelial disc. The discs were air dried, and another 15µl of supernatant was added. The total load per filter was finally 30 µl. PDA plates were incubated at 28±2°C for 7 days.

Spore suspension inoculation

The stock culture of *P. protegens* CHA0 ($OD_{600=0.6}$) was diluted to 1:100 with LB broth, which was supplemented with different concentrations of ANE (0% and 0.1%) and incubated for 14 days at 28±2°C and 180 rpm. The culture supernatant was collected after

centrifugation at 9600 rpm for 15 min. The resulting supernatant was then filtered through a syringe filter (VWR® syringe filters, PTFE, 0.2µm) to prevent the presence of bacterial cells. Clean LB broth was used as the control. FOC spore suspension (0.1mL) was spread uniformly on the surface of each PDA plate by using an L-shaped cell spreader. The filtered supernatants (15 µl) were spotted onto each sterilized filter paper disc (6 mm), which had been placed on each PDA plate 3cm away from each mycelial disc. The discs were air dried, and another 15 µl of supernatant was added. The total load per filter was finally 30 µl. PDA plates were incubated at 28±2°C for 2-3 days.

E.1.2 AGAR WELL DIFFUSION METHOD

The stock culture of *P. protegens* CHA0 ($OD_{600=0.6}$) was diluted to 1:100 with LB broth, which was supplemented with different concentrations of ANE (0% and 0.1%) and incubated for 14 days at 28±2°C and 180 rpm. The culture supernatant was collected after centrifugation at 9600 rpm for 15 min. The resulting supernatant was then filtered through a syringe filter (VWR® syringe filters, PTFE, 0.2µm) to prevent the presence of bacterial cells. Clean LB broth was used as the control. A sterile cork borer was used to construct three wells (5 mm) in each new PDA plate. FOC spore suspension was spread uniformly on the surface of each PDA plate. One well was filled with CHA0 supernatant, one well was filled with supernatant of CHA0 in 0.1%ANE, and the last well was filled with control (LB broth). PDA plates were incubated at 28±2°C for 2-3 days.

The diameters of inhibition zones for each disk and each well were measured as Figure E1. The inhibition percentage for the mycelial disc inoculation group was calculated as follow:

$$\text{Inhibition percentage}(\%) = \frac{(Rc - R_{treatment})}{Rc} \text{x}100$$

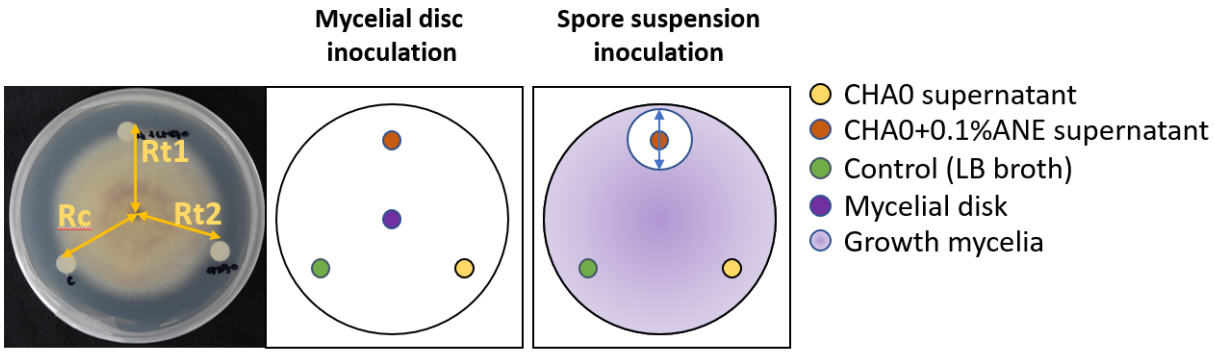

Figure E 1. Example plate layout for mycelial disc and spore suspension inoculation.

E.2 RESULTS

The cell-culture supernatants of *P. protegens* CHA0 cultured in LB broth and 0.1%ANE amended LB broth for one to 7 days did not exhibit significant antifungal activity (Table E1&Figure E2). We also did not observe the presence of a clear zone around the disk or the agar well in the supernatant of culture supernatants of *P. protegens* CHA0 cultured in LB broth and 0.1% ANE amended LB broth for 14 days.

Table E 1. Antifungal activity of *P. protegens* CHA0 cultured in LB broth and 0.1%ANE amended LB broth for one to seven days incubation by filter paper disc method.

Treatment	**Inhibition percentage (%)**						
	1 D	**2 D**	**3 D**	**4 D**	**5 D**	**6 D**	**7 D**
Control	0	0	0	0	0	0	0
CHA0	-3.3	-1.7	-0.5	1.3	2.2	2.6	4.4
CHA0+0.1%ANE	0.2	0.0	0.0	0.4	-4.4	3.1	3.2

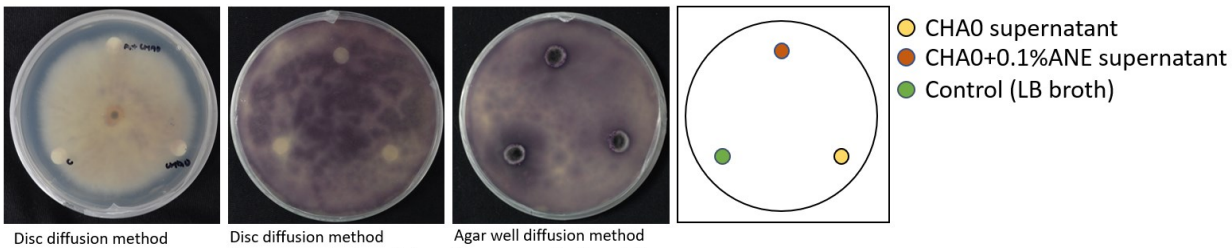

Figure E 2. Evaluation of the antimicrobial activity of culture supernatants of *P. protegens* CHA0 cultured in LB broth and 0.1%ANE amended LB broth against FOC.

APPENDIX F SEED TREATMENT *IN VIVO* TEST

F.1 METHODOLOGY

F.1.1 SOAK INOCULATION

Sterilized onion seeds were imbibed in SDW overnight for stratification. Then, seeds were soaked in FOC spore suspension (1% CMC, 10^6 spores/mL) or 1% CMC solution for one to two hours for the with pathogen inoculation group and the without pathogen inoculation group, respectively. Soaked seeds were then air dried in a laminar flow hood. Seeds (14 seeds per Petri dish) were sown in each 15 mm Petri dish (100 mm*15 mm) which was filled with around 18g Pro-mix BX (without mycorrhizae). Each treatment solution (Table F1) was evenly added to each Petri dish (20mL per Petri dish).

F.1.2 SOIL INOCULATION

Sterilized onion seeds were soaked in each treatment solution (Table F1) containing 1% CMC overnight. Soaked seeds were then air dried in a laminar flow hood. Inoculum suspension (FOC suspension) was mixed into Pro-mix (10^5 spores/g of Pro-mix) and then distributed into each Petri dish. Then, treated onion seeds (14 seeds per Petri dish) were sown in each Petri dish. For the without pathogen inoculation group, the same volume of SDW as the spore suspension was mixed into Pro-mix.

All Petri dishes were placed in a growth chamber, which was adjusted to 22±2°C (day/night) with a photoperiod of 16 h (cool white fluorescent tube lights, 150 µmol m^{-2} s^{-1}). Water was added daily to each Petri dish.

F.2 RESULTS

We observed that pathogen-uninoculated seeds germinated gradually, while pathogen-inoculated seeds in both soak and soil inoculation groups rarely or did not germinate.

Table F 1. *E. cloacae* CAL2 and ANE alone or their combination are evaluated for their efficiency for seed treatments *in vivo* test.

	Treatments	**Concentration of ANE (%)**	**Concentration of *E. cloacae* CAL2 (c.f.u./mL)**	**Other**
With pathogen and without pathogen inoculation	Control	-	-	water
	ANE	0.001	-	
		0.01	-	
		0.1	-	
	E. Cloacae CAL2	-	10^8	
	E. Cloacae CAL2+ANE	0.001	10^8	
		0.01	10^8	
		0.1	10^8	

APPENDIX G THE FINAL SEQUENCE OF FA AND FB

Fa: 477bp

CCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGG
ATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTC
TGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAAC
AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG
CACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCCCAAATTGATTGGCG
GTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGT
CGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTA
GGA

Fb: 467bp

ACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCT
CCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAAACTCTGTTTCTATA
TGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT
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GTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTCG
AGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCAC
GCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGA

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