Management of Fusarium Basal Rot Disease of Onion (*Allium cepa* L.) by Using Plant Growth Promoting Rhizobacteria in Seaweed Formulation

Report Year 2

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Submitted by

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PROJECT BACKGROUND

Onion (*Allium cepa* L.) is the second most important vegetable crop after tomato. The production of onions in Canada is around 251 thousand metric tonnes, with a market value of $110 million (Statistics Canada, 2019). The onion has considerable commercial importance due to its edible value and medicinal properties (Van Wyk, 2014). More than 570 acres of onions are grown in Nova Scotia (http://horticulturens.ca/). Onion production is generally affected by diseases caused by fungi and bacteria. The Fusarium basal rot (FBR) of onion, which is caused by 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 infection can occur at any stage of the onion plant's growth, causing significant damage both in the field and in storage. (Cramer, 2000; Taylor et al., 2019). Current methods, such as crop rotation, resistant cultivars usage, and fungicides application, are less effective in controlling the FOC (Coşkuntuna & Özer, 2008; Cramer, 2000; Rout et al., 2016). Therefore, the development of a new eco-friendly and viable approach to control onion FBR disease is of great necessity.

Biological control of plant diseases has been receiving considerable 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). Previous studies showed that the application of *Ascophyllum nodosum* extract reduced fungal disease in carrot and cucumber. (Jayaraj et al., 2008; Jayaraman et al., 2011). Plant growth-promoting rhizobacteria (PGPR) such as *Bacillus*, Enterobacter, *Paenibacillus*, and *Pseudomonas* have gained particular attention given their ability to control plant diseases and promote plant growth (Basu et al., 2021). PGPR can control plant diseases by synthesizing antibiotic metabolites and eliciting induced systemic resistance (Baehler et al., 2005; Ma et al., 2007; Maurhofer et al., 1994).

Therefore, the goal of this study is to evaluate the efficacy of *A. nodosum* extract and PGPR, singly and in combination, in controlling the FBR of the onion seedlings.

PROJECT STATUS UPDATES AND PROGRESS

Pathogenicity test of isolated FOC

Two isolates of pathogen FOC (Fig. 1) from onion fields (Berwick, Nova Scotia) were tested for pathogenicity on onion by using bulb, seedling, and pot pathogenicity tests.

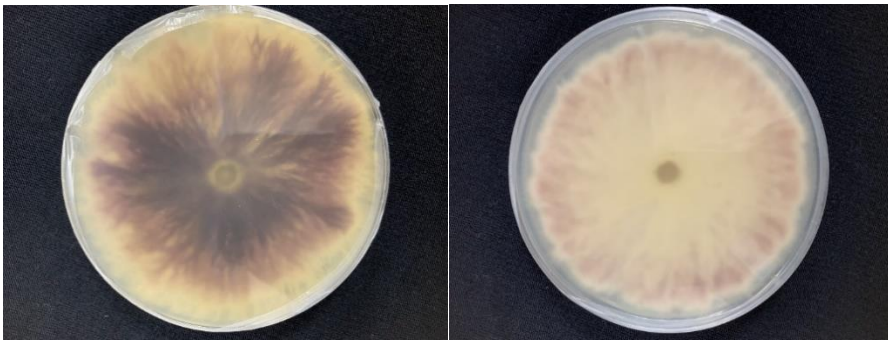


Figure 1. Two isolated of FOC from naturally infected onion bulbs (Fa-left, Fb-right).

For bulb pathogenicity test, FOC spore suspension (0.5 mL, 10^5 spores/mL) and 0.5 ml sterilized distilled water (SDW- control) were injected into the basal region of each surface-sterilized onion bulb (Yellow Dutch). The onion bulbs were incubated in the dark at 28±2°C, and disease symptoms were photographed one week after inoculation. It was observed that onion mature bulbs developed symptoms one week after inoculation. The Fa-inoculated group developed mild symptoms of basal rot including brown discoloration and rot at the basal plate and inner fleshy scales. The symptoms of the Fb-inoculated group were weak and hardly visible (Fig. 2).

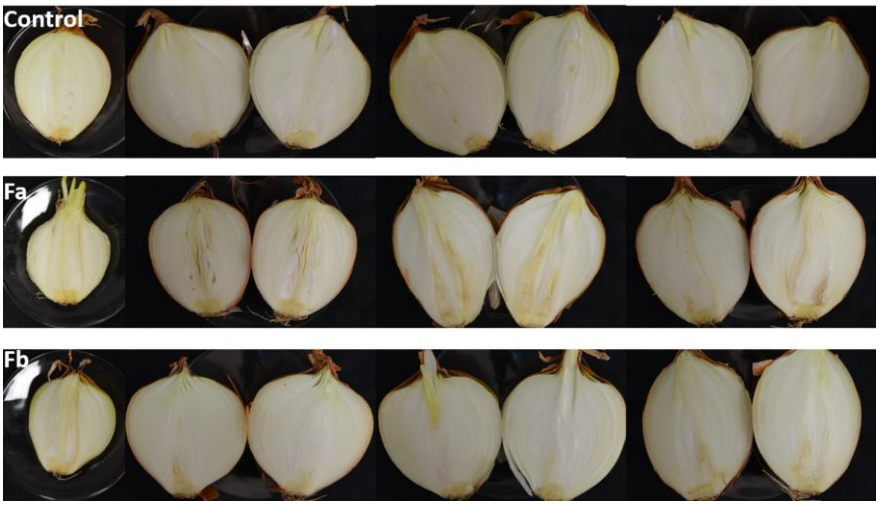


Figure 1. Symptoms of inoculated onion bulbs.

For the seedling pathogenicity test, the onion (Utah Sweet Spanish) seeds were sterilized using sodium hypochlorite and soaked overnight in SDW. Onion seeds were germinated on sterile Whatman paper in the Petri dish (15 seeds/ Petri dish). The seeds were inoculated with either 6 mL of SDW (control) or SDW6 mL FOC spore suspension (10^5 spores/mL) and incubated at 28±2 °C. SDW was added to each plate every three days to maintain moisture. After 9 days, the seeds were photographed, and the germination percentage was measured. The experiment was replicated thrice with four internal replicates. The results showed that the lowest germination percentage was observed in Fb group (52.8%), although there was no significant difference between Fa group (61.7%) and Fb group (Fig. 3).

For the pot pathogenicity test, onion sets (Yellow Dutch) were germinated on wet cheesecloth for one week and then inoculated with each of two FOC isolated spore suspensions (10^5 spores/mL) by root dipping for 30 minutes. The roots dipped in the SDW were used as a control. Then, onion plants were transplanted to the pots (three plants per pot). The biomass was collected one month after inoculation and data was represented as gram FW/plant. The experiment was replicated twice with six internal replicates. It was observed that the Fa-inoculated group had the lowest shoot biomass (2.91g/plant) while the Fb-inoculated group had the highest root biomass (0.34g/plant), but there was no significant difference among control, Fa-inoculated, and Fb-inoculated plants (Fig. 4). The transverse and longitudinal cross-sections of the lactophenol blue-stained onion root showed fungal invasion in onion root tissues (Fig. 5).

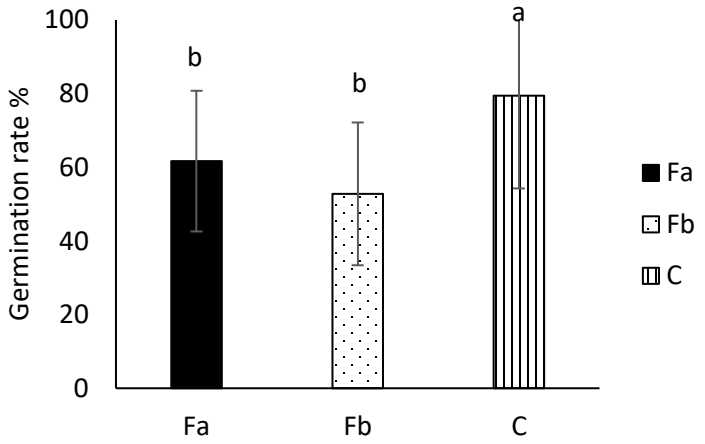


Figure 2. Germination rate of onion seeds inoculation with two FOC isolates (Fa and Fb) and control (C-SDW). Data was analyzed using One-Way ANOVA. Grouping was done using Turkey pairwise comparison and 95% confidence.

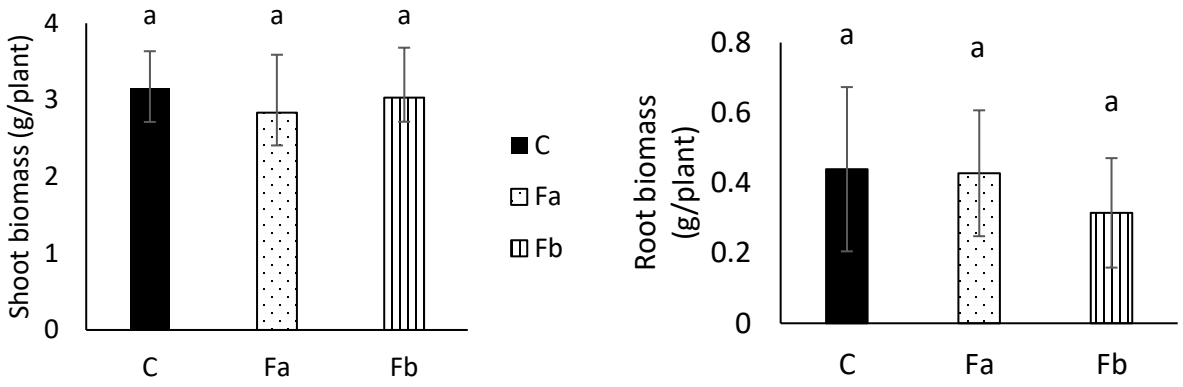
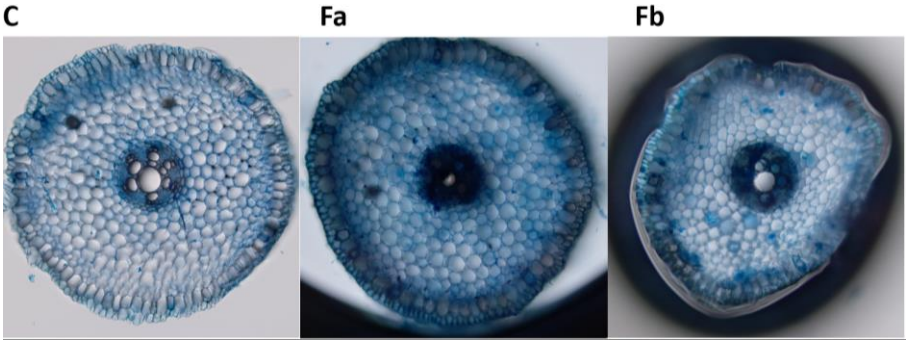


Figure 3. Biomass data (Fresh shoot weigh-left, Fresh root weight-right) of onion plants inoculated with two FOC isolates (Fa and Fb) and control (C-SDW). Data was analyzed using One-Way ANOVA. Grouping was done using Turkey pairwise comparison and 95% confidence.

a. Onion root cross sections.



b. Onion root longitudinal sections

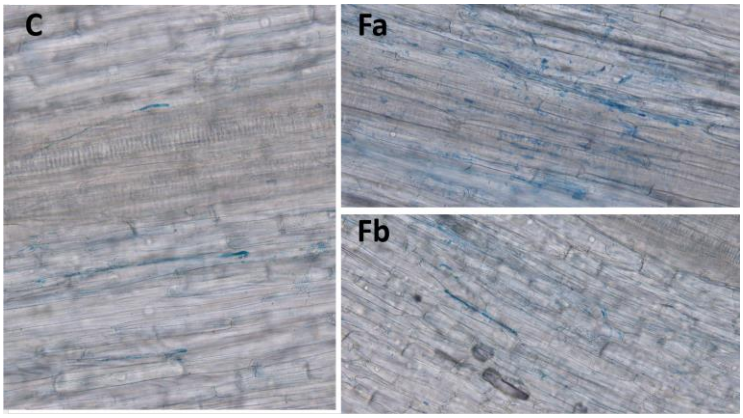
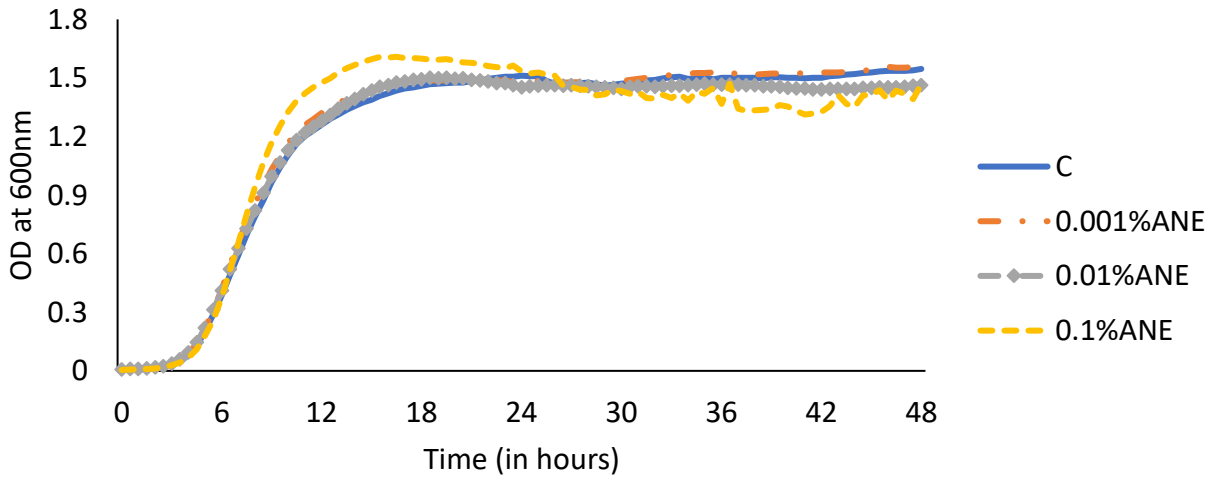


Figure 5. The lactophenol blue stained onion root sections observed under microscopy.

Evaluation of the effect of ANE on the growth of three PGPR strains (*Pseudomonas protegens* CHA0, *Bacillus subtilis*, and *Enterobacter cloacae* CAL2).

A spectrophotometric assay was conducted to examine whether ANE improved the growth of PGPR. The experiment was repeated thrice with three internal replicates. The bacterial growth curve was obtained by measuring changes in the optical density (OD). Bacteria was cultured in nutrient broth supplemented with different concentrations of ANE (Acadian Seaplants Limited) (0%-control, 0.001%, 0.01%, and 0.1%). The inoculated broths and blanks were subsequently plated as needed into a 96-well microplate and then incubated in a CytationTM 5 (BioTek, Winooski, VT) cell imaging multimode plate reader at 28±2˚C with linear shaking at 567 cpm (3 mm) for 24-48h. The OD was taken at 600 nm every 30 min. As shown in Fig. 6, the initial growth of PGPR strains was similar to that of control, and they reached the logarithmic phase after 4-6h. However, during the exponential phase, *P. protegens* CHA0 cultured in 0.1% ANE modified broth showed higher growth than other treatments, *B. subtilis* cultured in 0.01% and 0.1%ANE group were critically lower than the control group, and *E. cloacae* CAL2 cultured in 0.1% and 0.001% ANE modified broths showed higher growth than others. After reaching the stationary phase, there was no significant difference between the treatments for each PGPR strain. Therefore, a specific concentration of ANE can enhance the growth of some PGPR strains during the exponential phase.

a.



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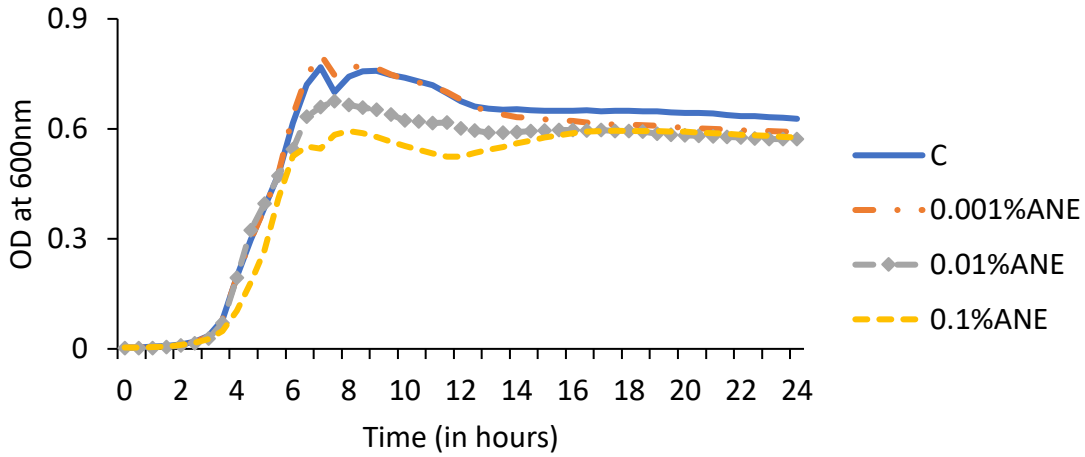

c.

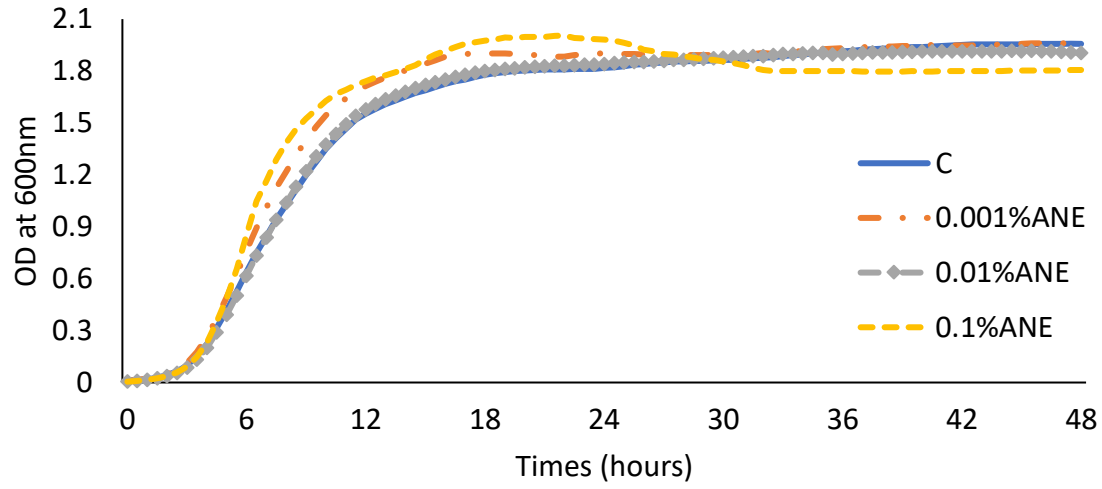

Figure 6. Effect of different concentrations of ANE on the growth of each PGPR. a. *P. protegens* CHA0; b. *B. subtills*; c. *E. cloacae* CAL2.

Effect of PGPR strains (*P. protegens* CHA0, *B. subtilis*, and *E. cloacae* CAL2) and ANE on the mycelial growth and spore germination of FOC

Dual culture assay and conidial germination assay were conducted to evaluate the effect of three PGPR strains and ANE on the mycelial growth and spore germination of FOC, respectively. Each experiment was repeated thrice with three replicates. For dual culture assay, each PGPR was inoculated to fresh broth supplemented with different concentrations of ANE (0%-control group and 0.1%), and incubated for 10h (28±2 ˚C, 180 rpm). Then, 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. Agar plug (5 mm) of FOC was inoculated 4 cm away from the bacterial

inoculation. 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 according to the following equation (Muniroh et al., 2019): Inhibition percentage (%) $= \frac{R_c - R_t}{R_c} \times 100\%$ (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). The results showed that the PGPR strains alone or in combination with 0.1% ANE exhibited significant pathogen inhibition compared to FOC grown alone (Table. 1). Among these, *E. cloacae* CAL2 inhibited the mycelial growth of FOC by 50% compared to the control group (Table.1c). *P. protegens* CHAO (22.2%) and 0.1% ANE combined with *P. protegens* CHAO (21.9%) exhibited moderate inhibitory activity against the mycelial growth of FOC (Table. 1a), and *B. subtilis* (13.8%) and 0.1% ANE combined with *B. subtilis* (12.9%) showed the lowest inhibitory activity against the mycelial growth of FOC (Table. 1b). However, there was no significant difference (P>0.05) between single PGPR treatment and PGPR combined with 0.1%ANE treatment.

Table 1. Dual culture assay of each PGPR with/without ANE against the pathogen mycelial growth. a. *P. protegens* CHA0; b. *B. subtills*; c. *E. cloacae* CAL2. Data was analyzed using One-Way ANOVA. Grouping was done using Turkey pairwise comparison and 95% confidence.

a.

Treatment	**Inhibition%± D**	
CHA0	22.2±8.12	a
CHA0+0.1%ANE	21.9±8.92	a
Control	0±5.26	b

b.

Treatment	**Inhibition%±SD**	
BS+0.1%ANE	13.8±1.81	a
BS	12.9±2.78	a
Control	0±0	b

c.

Treatments	**Inhibition%±SD**	
CAL2	56.3±2.53	a
CAL2+0.1%ANE	54.1±3.83	a
Control	0.00±2.15	b

For conidial germination assay, 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 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 germination was observed in five different views under the microscope. Total 100 spores and the number of germinated spores among those spores were randomly counted and recorded in each view. The germination rate was calculated as follow: $\text{Germination rate } (\%) = \frac{\text{Total counted germinated spores in five views}}{\text{Total counted spores in five views}} \times 100\%$. The results were similar to the dual culture assay. 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 (Table. 2). *E. cloacae* CAL2 (9.93%) and 0.1% ANE combined with *E. cloacae* CAL2 (13.2%) strongly inhibited the spore germination of FOC. *P. protegens* CHAO (36.7%) and 0.1% ANE combined with *P. protegens* CHAO (30.6%) group was the next most effective, and *B. subtilis* (40.6%) and 0.1% ANE combined with *B. subtilis* (52%) showed the lowest inhibitory activity (%) against the spore germination of FOC. ANE treatment showed no significant effect on the spore germination of FOC. Furthermore, there was no significant difference (P>0.05) between single PGPR treatment and PGPR combined with 0.1%ANE treatment.

Table 2. Inhibitory effect of each PGPR and ANE on spore germination of the pathogen. a. *P. protegens* CHA0; b. *B. subtills*; c. *E. cloacae* CAL2. Data was analyzed using One-Way ANOVA. Grouping was done using Turkey pairwise comparison and 95% confidence.

a.

Treatment	Germination rate%±SD	
Control	73.9±5.56	a
ANE	68.8±5.84	a
CHA0	36.7±8.21	b
CHA0+ANE	30.6±4.81	b

b.

Treatment	Germination rate%±SD	
ANE	75.5±9.24	a
Control	72.2±10.66	a
BS+ANE	52.0±10.05	b
BS	40.6±12.27	b

c.

Treatment	Germination rate%±SD	
ANE	92.6±1.11	a
Control	90.0±1.56	a
CAL+ANE	13.2±2.95	b
CAL	9.93±1.70	b

Evaluation of *Ascophyllum nodosum* extract formulation + *P. protegens* CHA0 for the management of basal rot and soft rot of onion crop in the greenhouse and field conditions.

Two greenhouse experiments were conducted to study the combined effects of ANE and *P. protegens* CHA0 on basal rot development. Each experiment had four replicates and each replicate comprised 10 pots with three plants in each pot. Onion seeds were sterilized using sodium hypochlorite and soaked overnight in water. The soaked seed was planted in a tray filled with Promix and placed in the greenhouse. Six weeks old plants grown from seed were used in the experiments. ANE was used at a concentration of 0.1%. *P. protegens* CHA0 was cultured in King's B medium, and 10^8 cell/ml bacterial cell suspension was used in the experiment. The pathogen inoculation was done using the root dip method. *F. oxysporum* f. sp. *cepae* was grown on potato dextrose agar (PDA) medium for 10 days and spore suspension was collected by flooding the Petri dish with SDW. The spore suspension was passed through sterile glass wool to remove mycelial fragments. A suspension of 1.17×10^8 spores/mL was made in SDW and inoculated by root drench method 24 h after the ANE treatment. Root sample was collected for biochemical and molecular studies at 3-time points; 24 h, 48 h, and 72 h post-inoculation. Fifteen days after the inoculation of plants with *F. oxysporum* f. sp. *cepae* it has been determined that none of the treatments stopped the infection, implying that disease incidence was the same in control and treated plants. However, differences were observed in disease severity; disease progression was much slower in treated plants resulting in better growth and higher biomass (Fig. 7).

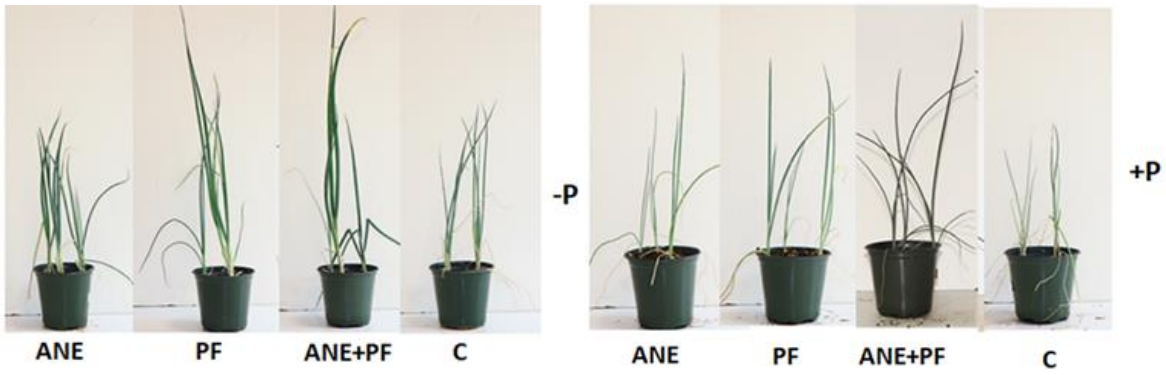

Figure 7. Phenotype of the plants at 15 days after the infection with *F. oxysporum* f. sp. *cepae.* ANE, *A. nodosum* extract; P, *P. protegens* CHA0; P; *F. oxysporum* f. sp. *cepae*; C, control.

In order to visualize the initial stages of infection, root sections (2 cm) were stained with lactophenol blue and observed under a microscope to assess pathogen development. Quantification

of the pathogen was carried out by extracting the DNA from roots followed by real time quantitative PCR (RT-qPCR) using the standard curve method and specific primers targeting *Fusarium* genomic ITS region. Cytological observations revealed less mycelium density in treated plants in compared to control (Fig. 8a). Quantification of the pathogen by RT-qPCR revealed that all treatments inhibited pathogen development in compared to the control (Fig. 8b). The lowest number of *Fusarium* cells (2.06×10^3 cells/g FW) was observed in PF-treated plants followed by ANE+PF (2.08×10^3 cells/g FW), ANE (5.10×10^3 cells/g FW) and control (6.71×10^3 cells/g FW) (Fig. 8 b).

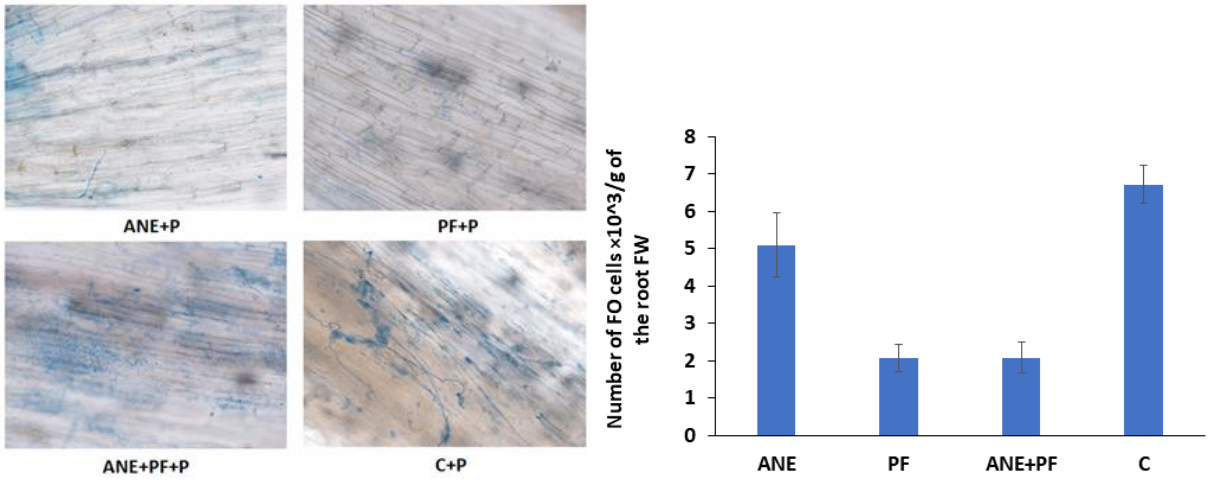

Figure 8. **a.** Pathogen development in the onion root. **b.** Quantification of the pathogen in the root. ANE, *A. nodosum* extract; P, *P. protegens* CHA0; P; *F. oxysporum* f. sp. *cepae*; C, control.

Evaluation of biochemical changes in the onion plant and the mode of action of seaweed + biocontrol agent formulation.

In order to investigate the mechanism(s) by which ANE and *P. protegens* CHA0 suppress the development of the pathogen in onion, the activity of defense related enzymes was studied. The activity of the phenylalanine ammonia lyase (PAL), peroxidases (Prx) enzymes as well as hydrogen peroxide (H_2O_2), and total phenolic content (TPC) was quantified.

Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway and is induced under a variety of biotic and abiotic stress. PAL is a key regulator of the phenylpropanoid pathway, which includes the biosynthesis of a wide array of polyphenol compounds such as flavonoids, phenylpropanoids, and lignin, compounds that are essential for growth and response to pathogen attack. As is seen in Fig.9, in the absence of the pathogen PAL activity was found to gradually increase from 24 h to 72 h in all treatments (Fig. 9). ANE+PF was

found to activate PAL better than when ANE and PF used separately. PAL activity was further increased in all treatments after pathogen inoculation. The highest PAL activity (32.13 nmol cinnamic acid/min/mg protein) was observed in the plants treated with ANE and PF after pathogen challenge (Fig. 9).

Peroxidases (Prx) are key antioxidant enzymes involved in numerous physiological processes in plants, involving responses to abiotic and biotic stresses, defense mechanisms, and the biosynthesis of lignin. ANE and PF did not enhance Prx activity in the absence of the pathogen, and no clear trend could be determined at different time points (Fig. 10). In presence of the pathogen, much stronger responses were observed at 24h, in all conditions, except for ANE + PF after 72h of pathogen challenge, the point at which the highest PO activity (5.63 U/min /g FW) was determined (Fig. 10). Overall, the priming effects of peroxidases activity by ANE, PF, or of their combination was not obvious; it remains to be determined in future experiments if Prx response is dependent on the concentration of ANE and the number of PF cells.

Hydrogen peroxide (H_2O_2) is produced during oxidative stress and plays a crucial role in the defense response against pathogen challenges. No clear changes were observed after ANE, PF, and ANE + PF treatments (Fig. 11). An early response resulting in H_2O_2 accumulation was observed, 24 h after the pathogen challenge, in control and all treatments. Notably, stronger responses were determined to occur in presence of treatments as compared to control; the highest accumulation was found 27.36 in ANE+PF treated plants, 24 h after the pathogen challenge (Fig. 11). The production of phenolic compounds is derived from the phenylpropanoid pathway, so the total phenolic content (TPC) is an important index to assess the functionality of this pathway that plays key roles in plant defense mechanisms. Also, TPC allows assessing the redox capacity, which is essential in the response of plants to pathogens. In the absence of pathogen, TPC was found to be consistently higher in PF followed closely by ANE + PF and ANE (Fig. 12). Interestingly, the timing of activation was quite different, the highest amounts of TPC were found in PF and ANE at 48 h while in ANE+PF at 24 h. Variability in the time points was also observed in the presence of the pathogen; a higher TPC accumulation was observed 48 h after the pathogen challenge. Among treatments, the highest TPC accumulation (38.9µg/g of FW) was observed in PF-treated plants, at 48 h from pathogen inoculation (Fig. 12).

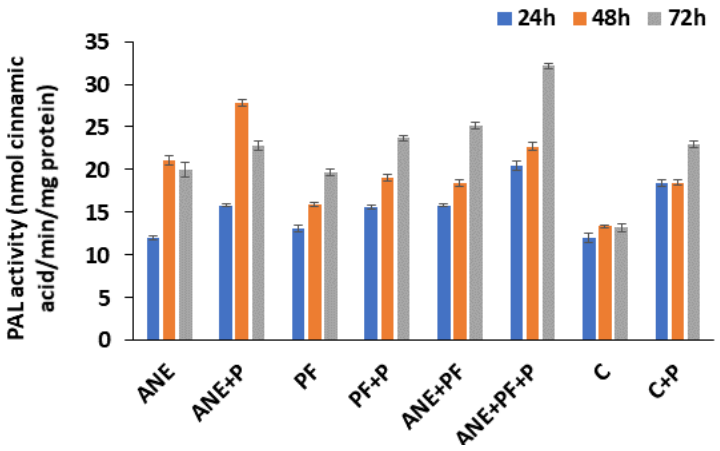

Figure 9. Phenylalanine ammonia lyase (PAL) activity in onion roots at 24 h, 48 h and 72 h after the infection with *F. oxysporum* f. sp. *cepae.* ANE, *A. nodosum* extract; P, *P. protegens* CHA0; P; *F. oxysporum* f. sp. *cepae*; C, Control.

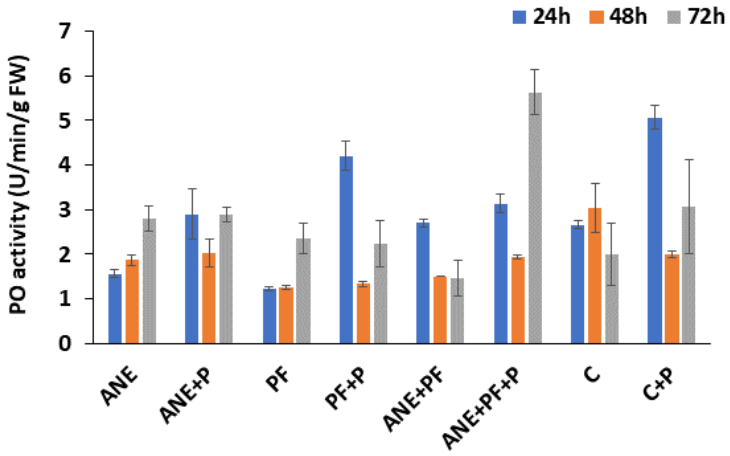

Figure 10. Peroxidase (Prx) activity in onion roots at 24 h, 48 h and 72 h after the infection with *F. oxysporum* f. sp. *cepae.* ANE, *A. nodosum* extract; P, *P. protegens* CHA0; P; *F. oxysporum* f. sp. *cepae*; C, Control.

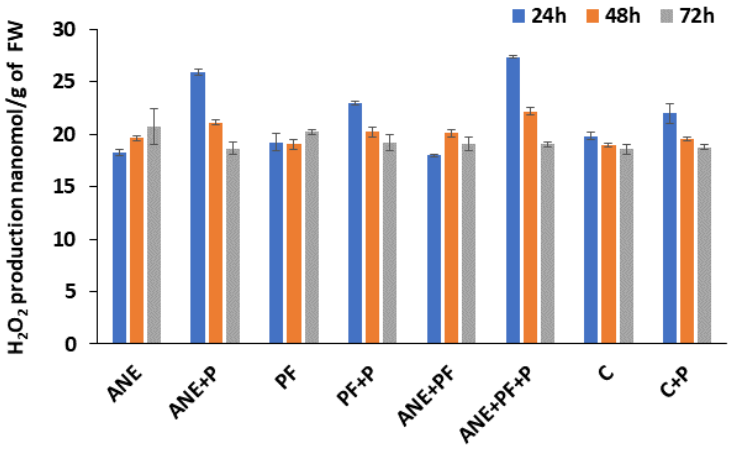

Figure 11. Production of hydrogen peroxide (H_2O_2) in onion roots at 24 h, 48 h and 72 h after the infection with *F. oxysporum* f. sp. *cepae.* ANE, *A. nodosum* extract; P, *P. protegens* CHA0; P; *F. oxysporum* f. sp. *cepae*; C, Control.

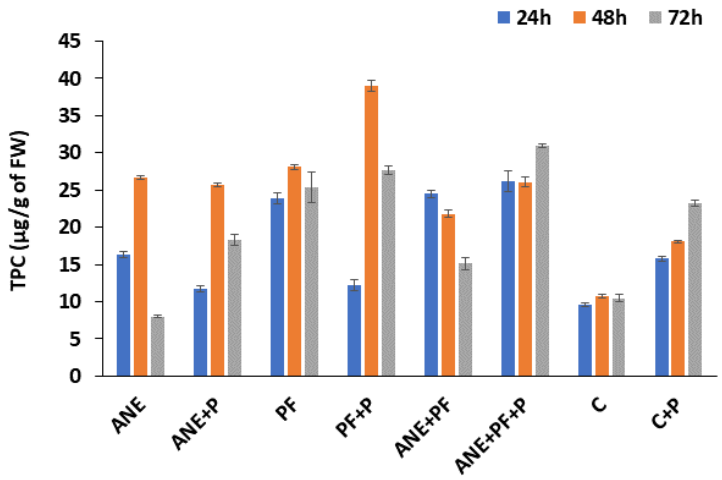

Figure 12. Total phenolic content (TPC) in onion roots at 24 h, 48 h and 72 h after the infection with *F. oxysporum* f. sp. *cepae.* ANE, *A. nodosum* extract; P, *P. protegens* CHA0; P; *F. oxysporum* f. sp. *cepae*; C, Control.

Conclusion

The study proved that three Plant Growth Promoting Rhizobacteria (*Pseudomonas protegens* CHA0, *Bacillus subtilis*, and *Enterobacter cloacae* CAL2) had an antifungal activity against onion fusarium basal rot pathogen *Fusarium oxysporum* f. sp. *Cepae*. under in-vitro condition. ANE did not show any antifungal activity, and the addition of ANE did not enhance but neither weaken the antifungal activity of three PGPR strains under the in-vitro condition. However, we observed that a specific concentration of ANE can enhance the growth of some PGPR strains. ANE, PF and ANE+PF treatments improved resistance to *F. oxysporum* f. sp. *cepae* by priming the plants before fungal infection. Generally, ANE+PF provided better protection than the individual treatments. We plan to continue with greenhouse experiments to assess the effect of seaweed and *Enterobacter cloacae* CAL2 as it showed the highest antifungal activity under *in-vitro* condition. Field experiments could not be carried out during 2021 crop season due to COVID-19 restrictions, we are planning to do field trials during 2022 season.