UNIVERSIDADE D
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Bichitra Paul

CHARACTERISATION OF THE CYTOKININ MEDIATED BIOCONTROL ACTIVITY OF *CHLAMYDOMONAS REINHARDTII* AGAINST *PSEUDOMONAS SYRINGAE* IN TOBACCO AND ANALYSIS OF ITS POTENTIAL AS A BIO-STIMULANT

Dissertação no âmbito do Mestrado em Ecologia orientada pelo Professor Doutor Thomas Georg Roitsch e pela Doutora Joana Cardoso da Costa e apresentada Departamento de Ciências da Vida da Facultade de Ciências e Tecnologia da Universidade de Coimbra.

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Resumo

As microalgas são um grupo grande e diversificado de microrganismos que desempenham um papel importante na agricultura onde são utilizadas como biofertilizantes. Estudos anteriores demonstraram que as microalgas têm diversos efeitos sobre as plantas, como a indução de uma germinação mais rápida, promoção do crescimento de folhas e raízes e indução da resistência a organismos patogénicos quando aplicadas no solo. As microalgas também têm a capacidade de produzir fitohormonas incluindo auxina, ácido abscísico (ABA), citocinina (CK), etileno (ET) e giberelinas (GAs).

As CK são uma classe bem conhecida de fitohormonas que desempenham um papel importante em várias funções das plantas ao longo da vida. Estes incluem o crescimento da raiz ou do rebento, a divisão celular e a senescência. Além disso, estudos recentes demonstram que as CKs também desempenham um papel significativo na defesa das plantas tendo sido registado um claro aumento das defesas contra bactérias patogénicas induzidas por microrganismos produtores de CK. De facto, foi relatado que o pré-tratamento de plantas com CKs produzidas pela estirpe G20-18 de *Pseudomonas fluorescens* mitigou a infeção por bactérias patogénicas.

O objectivo geral deste estudo foi investigar se a estirpe da microalga *Chlamydomonas reinhardtii*, produtora de CKs, poderia promover a resistência à bactéria patogénica *Pseudomonas syringae* em *Nicotiana tabacum*. Em paralelo, testou-se ainda se a mesma microalga poderia atuar como bioestimulante nutras espécies vegetais. Para atingir estes objetivos foram realizadas várias experiências em que se utilizaram duas estirpes selvagens e seis estirpes mutantes de *C. reinhardtii* (knockouts dos genes que codificam a LOG e HMBPP redutase envolvidos na produção e sinalização de CK).

Os resultados obtidos evidenciaram uma forte promoção da resistência de *N. tabacum* ao agente patogénico após infiltração com estirpes selvagens de *C. reinhardtii*. Por outro lado, as estirpes mutantes deficientes na produção e sinalização de CKs evidenciaram uma redução na promoção dessa resistência. Estes resultados comprovam que as CKs são uma parte essencial da capacidade que as microalgas têm de promover a resistência a agentes patogénicos. Em paralelo, demonstrou-se ainda que estirpes produtoras de CKs promovem o crescimento de plantas de tomateiro, tabaco e *Arabidopsis* suportando o seu papel como bioestimulante. Por fim, obtiveram-se evidências de que estas estirpes também podem estar envolvidas na indução da tolerância à seca em *Arabidopsis*.

A utilização em paralelo neste trabalho de estirpes de *C. reinhardtii* produtoras de CKs e estirpes mutantes incapazes da sua produção, permitiu concluir que a capacidade de biocontrolo, bioestimulante e tolerância à seca induzida pelas estirpes selvagens se deva à produção de CKs. Estes resultados constituem uma evidência preliminar de que a microalga *C. reinhardtii* pode constituir um potencial concorrente aos fertilizantes e produtos utilizados na agricultura convencional, e que num futuro próximo pode ser utilizada como uma solução sustentável na bio-fertilização e biocontrol.

PALAVRAS-CHAVE: *Chlamydomonas reinhardtii*, citocinina, bio-fertilização, biocontrol.

Abstract

Microalgae are a large and diverse group of microorganisms that play an important role in agriculture where they are used as bio-fertilisers. Previous studies have shown that microalgae have several effects on plants, such as inducing faster germination, promoting leaf and root growth and inducing resistance to pathogens when applied to soil. Microalgae also have the ability to produce phytohormones including auxin, abscisic acid (ABA), cytokinin (CK), ethylene (ET) and gibberellins (GAs).

CKs are a well-known class of phytohormones that play an important role in various plant functions throughout life. These include root or shoot growth, cell division and senescence. In addition, there is evidence that CKs also play a significant role in plant defence. This evidence comes from recent studies in which there is a clear increase in defences against pathogenic bacteria induced by CK-producing microorganisms. In fact, it has been reported that pre-treatment with CKs produced by *Pseudomonas fluorescens* strain G20-18 has mitigated infection by pathogenic bacteria in plants.

The overall objective of this study was to investigate whether a CK-producing strain of the microalgae *Chlamydomonas reinhardtii* could promote resistance to the pathogenic bacterium *Pseudomonas syringae* in *Nicotiana tabacum*. In parallel, it was also tested whether the same microalgae could act as a bio-stimulant for other plant species. To achieve these goals, several experiments were carried out using two wild type strains and six mutant strains of *C. reinhardtii* (knockouts of genes coding for LOG and HMBPP reductase involved in the production and signalling of CK).

The results showed a strong promotion of N. *tabacum* resistance to the pathogen after infiltration with the wild type strains of *C. reinhardtii*. On the other hand, mutant strains deficient in the production and signalling of CKs showed a reduction in the promotion of this resistance. These results prove that CKs are an essential part of the ability of microalgae to promote resistance to pathogens. Furthermore, the results obtained also showed that CK producing strains promote the growth of tomato, tobacco and *Arabidopsis* plants - suggesting their role as bio-stimulants. Finally, evidence has been obtained that these strains may also be involved in inducing drought tolerance in *Arabidopsis*.

The use of both wild type and KO mutant strains of *C. reinhardtii* led to the conclusion that the biocontrol capacity, bio-stimulant and drought tolerance is due to the production of CKs. These results provide preliminary evidence that the *C. reinhardtii* may be a potential competitor to conventional agricultural fertilisers and products and that soon it may be used as a sustainable solution in bio-fertilisation and biocontrol.

KEYWORDS: *Chlamydomonas reinhardtii*, cytokinin, bio-fertilization, biocontrol

Contents

List of Abbrevations

- 045820 LMJ.RY0402.045820 KO mutant strain
- 200648 LMJ.RY0402.200648 KO mutant strain
- ABA Abscisic acid
- BRs Brassinosteroids
- CC 125 Cre CC-125 wild type *Chlamydomonas reinhardtii*
- CC 5325 Cre CC-5325 wild type *Chlamydomonas reinhardtii*
- Chl *Chlamydomonas reinhardtii*
- CK Cytokinin
- CLiP *Chlamydomonas* library project
- cLOG LMJ.RY0402.200648 KO mutant strain
- cZ *cis*-Zeatin
- D Drought – treated
- DMAPP Dimethylallyl diphosphate
- DPI Days Post Infection
- DZ Dihydrozeatin
- ET Ethylene
- GA Gibberellins
- HMBPP LMJ.RY0402.045820 KO mutant strain
- HMBPP reductase 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate reductase
- iP isopentenyl adedine
- IPP isopentenyl phosphate
- IPT isopentenyl transferase
- JA Jasmonic Acid
- KO Knockout
- LOG Lonely Guy Gene
- MEP Methylerythritol phosphate pathway
- MS Murashige and Skoog medium
- MVA Mevalonate pathway
- NT *Nicotiana tabacum*
- OD Optical density
- PH 144 PH 144 (2-LOG) CRISPR/Cas9 KO mutant
- PH 145 PH 145 (2-LOG) CRISPR/Cas9 KO mutant
- PH 146 PH 146 (2-LOG) CRISPR/Cas9 KO mutant
- PH 147 PH 147 (2-LOG) CRISPR/Cas9 KO mutant
- PstT *Pseudomonas syringae* pv. *tabaci*
- RPM Revolutions per minute
- SA Salicylic Acid
- SWC Soil Water Content
- tZ trans-Zeatin
- WT Wild Type
- WW Well-watered

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1.Introduction

Most plants do not have locomotor functions so they cannot escape stress situations, be they biotic or abiotic. Hence, they have evolved by developing highly regulated strategies involving a multitude of compounds to address such situations, namely attack by pathogens. Perhaps the most important of these compounds are the phytohormones (plant hormones), which play a crucial role in helping the plant to cope with stressful situations. These compounds are growth hormones, such as auxins, cytokinins (CKs), gibberellins (GA) and brassinosteroids (BRs) (Davies 1995). CKs are hormones which influence the plant throughout various stages of their life cycle, such as shoot initiation, cell division, leaf senescence, sink/source relationships, nutrient uptake, flower development, vascular proliferation, female gametophyte development, etc. (Roitsch, Ehneß 2000; Hwang, Sheen, Müller 2012; Kieber, Schaller 2014; Wybouw, Rybel 2019).

1.1 CK Biosynthesis

Chemically, CKs are adenine derivatives with distinguishable substitutions attached to the N^6 position of the adenine ring. Most naturally occuring CKs have isoprenoid side chains, isopentenyladedine (iP), trans-Zeatin (tZ), cis-Zeatin (cZ) and Dihydrozeatin (DZ) as illustrated in Figure 1.1 (Sakakibara 2006).

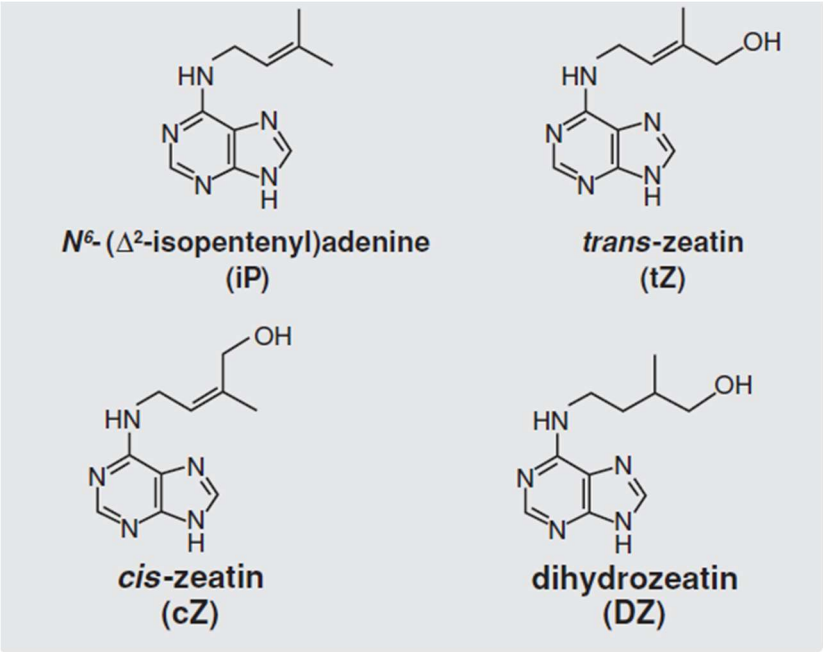


Figure 1.1: Structures of the naturally occuring isoprenoid CKs (Sakakibara 2006)

Even till now, the actual process of CKs biosynthesis has not been fully understood. However, it is almost universally accepted that there are two distinct pathways which play a role in it, namely the Methylerythritol Phosphate Pathway (MEP) and the Mevalonate Pathway (MVA).

In the MEP pathway, the hydroxymethylbutenyl diphosphate is converted to dimethylallyl diphosphate (DMAPP). Then the DMAPP is transformed to isopentenyladedine (iP), by isopentenyl transferase (IPT), and then to tZ and DZ (Sakakibara 2006; Chang, Song, Liu, Liu 2013; Kieber, Schaller 2014). Although tZ and cZ are isomers, there is almost no interconversion between the *cis* and *trans* forms of Zeatin (Gajdošová et al., 2011).

In the MVA pathway, acetyl CoA is converted to isopentenyl phosphate (IPP) and its isomer DMAPP, and while the initial general consensus was that the MVA pathway was the sole producer of CK precursors, many recent studies have shown that it is the MEP pathway that produces the bulk of CKs, and only cZ is produced by the MVA pathway (Figure 1.2) (Kasahara et al., 2004; Sakakibara 2006; Chang et al., 2013; Kieber, Schaller 2014).

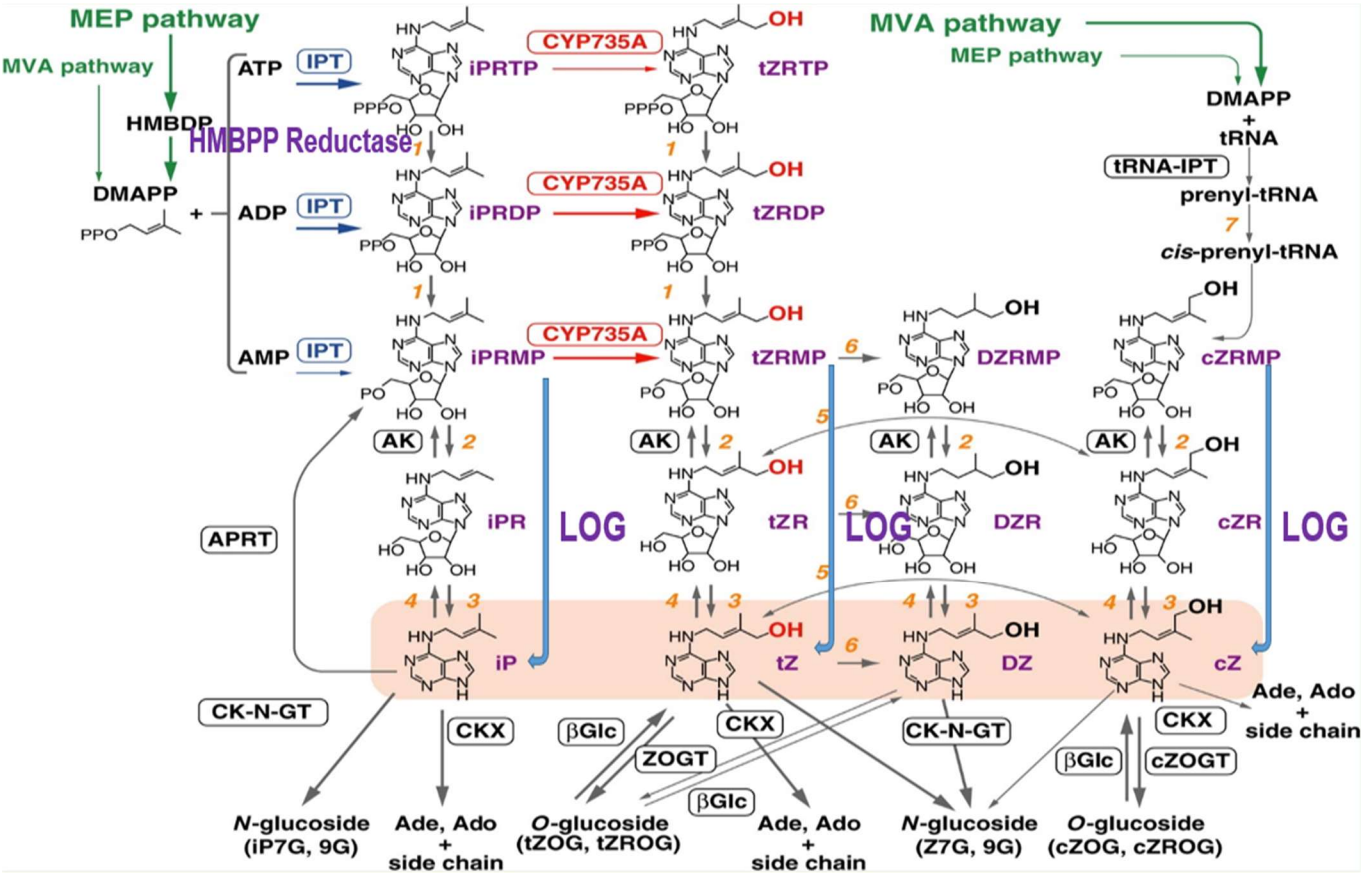


Figure 1.2: Current model of isoprenoid CK biosynthesis pathways in *Arabidopsis*. This shows that the isoprenoid sidechains of the iP and tZ originate predominantly from the MEP pathway, while a major fraction of the cZ chain comes from the MVA pathway (Adapted from Sakakibara, 2006).

1.2 Host-pathogen responses in plants via CKs

For a long time, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) were described to be the main players in the plant defence responses, but in the past decade a lot of studies have demonstrated that abscisic acid, GA, CK, auxin and BRs are also vital for the immune responses of plants, even though these hormones are mainly associated with abiotic stress or developmental processes (Pieterse et al. 2012; Shigenaga, Argueso 2016). In fact, it is now evident that it is a synergistic interaction between the various hormones that provides immunity to the plant, such as between CKs and ABA (Ha et al. 2012). This is since invading pathogens have different and unique strategies to infect plants, hence it only makes sense that this complicated interconnected mesh of different defence responses helps the plant to fight back these pathogens.

With respect to the host physiology, pathogens can be divided into biotrophic or necrotrophic pathogens. Biotrophic pathogens need the host plant cells to stay alive to derive its nutrients, while necrotrophic pathogens must kill its host to get nutrients from it (Glazebrook 2005). Interestingly, *Pseudomonas syringae* is a pathogen which is both biotrophic and necrotrophic. When it initially infects a leaf, it acts like a biotrophic pathogen, but *P. syringae* gradually kills the infected tissue, resulting in a necrotrophic pathogen. Hence, it is referred to as a hemi-biotrophic pathogen (Rico, Preston 2008).

1.3 CKs Role in Stress Responses

As already mentioned, CKs are a group of phytohormones that aid the plant in growth functions (Argueso, Ferreira, Keiber 2009). Recently, CKs have also been linked to biotic stress responses and involved in the defence strategy of plants against invading pathogens (Argueso et al. 2012). However, some studies which used exogenous CKs to investigate their role in biotic stress responses had mixed success (Clarke et al. 1998; Babosha 2009). Eventually, some experiments using *Arabidopsis* helped establish the role of CKs in plant pathogen resistance, where it was demonstrated that certain concentrations of CKs could reduce the growth of the biotrophic and hemi-biotrophic pathogens *Hyaloperonospora arabidopsis* and *P. syringae*, respectively. Once the experiments were dependent on CK receptors, they conclusively proved that it was in fact the CK-regulated physiological process that caused the resistance in plants (Choi et al. 2010; Argueso et al. 2012). In addition to this, a recent study has successfully demonstrated that application of CKs, or using a high CK-producing strain,

also stimulates the activation of a defence response against nematode infection, which further expands the scope of the plant pathogen interactions in which CKs are involved in (Shanks et al. 2016).

1.4 Hormonal Communication between plants and microorganisms

Plants are not standalone creatures since they interact with other living organisms. What is interesting is the spectrum of plant-microbe interactions ranging from positive to negative interactions, such as commensalism, mutualism and pathogenesis (or parasitism) (Ghosh, Chowdhury, Bhattacharya 2016).

For instance, the bacteria *Rhizobium* sp. sends a hormonal signal to the plant, which causes it to form a nodular formation on its roots that helps the plant to fixate nitrogen from the air (Child 1975). Biotrophic pathogens also communicate with the plant while living inside a part of it, such as in the leaf (Ciesielska et al. 2014). Plants and microorganisms communicate with each other by using hormones or effectors. These effectors produced by microorganisms can trigger hormonal responses in the plant, which in turn triggers another hormonal response in the microorganism and so on.

This evolutionary arms race between plants and pathogens has created a kind of "hormonal conversation" between them during the infection process (Jones, Dangl 2006). Different effectors from pathogens induce different responses in plants, as different species of plants react differently when introduced to the same pathogen (Verhage, van Wees, Pieterse 2010). For example, in *Arabidopsis* sp. resistance is induced through SA production (Choi et al. 2010), while in *Nicotiana tabacum* the defence responses are upregulated by the increased production of the phytoalexins scopoletin and capsidiol (Großkinsky et al. 2011).

It has also been demonstrated that some microorganisms synthesize CK in order to resemble the CKs from the plant, or might even utilise the CKs produced by the plants for their own gain (Greene 1980). The CKs receptors in the plants bind to both plant and microorganism produced CKs, but they result in different molecular outputs (Ciesielska et al. 2014). Many biotrophic pathogens utilise this CK communication tool to promote unusual development or symptoms in plants for their own benefit. For example, the part of the leaf that is infected can "stay alive" longer than its surrounding tissue, a phenomenon referred to as "green islands" on dead leaves (Walters, McRoberts 2006). CKs production by pathogens can also lead to gall formation, which is an essential aspect for the lifestyle of many biotrophic pathogens (Hwang, Sheen, Müller 2012; Naseem, Kaltdorf, Dandekar 2015). This nutrient mobilisation effect that CKs have on plant tissue creates sinks in the "sink-source"

relationship of sugars in plants. This is why the CK production by many pathogens is an imperative tool for the invasion of pathogens on the desired tissue (Roitsch, González 2004; Choi et al. 2010).

1.5 The role of beneficial microbes in host responses to pathogens

Several microorganisms have been described as beneficial for plants (Kumar, Karthikeyan, Prasanna 2016). An example of this positive effect is the promotion of growth, such as the different types of rhizobacteria that have a symbiotic relationship with plants helping them to obtain nutrients from the soil (Ciesielska et al. 2014). Other examples of such interactions are biological nitrogen fixation from the air, bacterial enhancement of phosphate availability and also the bacterial production of phytohormones, such as auxins and CKs, that increase plant growth (Ortíz-Castro, Valencia-Cantero, López-Bucio 2008). Diseases caused by pathogens can be mediated in plants through a wide range of mechanisms. For instance, in soil, beneficial microbes can compete for nutrients in a way that inhibits the growth of pathogens; also some beneficial microbes may directly inhibit the growth of pathogens by excreting antifungal and antibiotic products, while in some cases the growth of beneficial microorganisms can induce resistance towards a potentially invading pathogen (Grondona et al. 1997; Sarma et al. 2002; Lucas et al. 2009; Bashan et al. 2014).

1.6 Phytohormone production in beneficial microbes

Many recent studies have delved deeper into understanding the mechanisms by which these beneficial microbes provide this biological resistance to plants. For example, the pre-treatment of plants with these microorganisms resulted in a form of priming of the defence responses. By priming the plant for an imminent infection, the plant can easily overcome the pathogen attack and as a result the plant is not affected as badly (Haas, Défago 2005). Many of these beneficial microbes that prime the plant achieve this effect by using phytohormones (Wu et al. 2009; Park et al. 2017). What is fascinating is that several microorganisms prime the plant by using CKs (Wu et al. 2009; Marimuthu et al. 2013). A recent study showed that injecting a CK producing strain of *Pseudomonas fluorescens* G20-18 in leaves of *Arabidopsis thaliana* induced pathogenic resistance in the plant. For further confirmation, this bacterial strain was genetically altered in order to halt CK production, and it was observed that this altered strain did not provide the same resistance as its predecessor. Then, a “regain of function”

mutant was created, and this mutant restored the immunity against invading pathogens. Thus, this evidence suggests that in this bacterial strain CKs caused the observed promotion in resistance in plants (Großkinsky et al. 2016).

1.7 Bio-stimulant and bio-fertiliser activity of microalgae

Microalgae are microscopic photosynthetic organisms that live in both marine and freshwater environments, as well as in soil, so a lot of research has also been going on to address the beneficial role of microalgae in soil (Babu et al. 2015; Garcia-gonzalez, Sommerfeld 2016; Ghaderiardakani et al. 2019; Kholssi et al. 2019). The term microalgae refer to both eukaryotic and prokaryotic microorganisms that have a photosynthetic apparatus. For a long time, the use of macroalgae as bio-fertiliser in agricultural fields was a well-known practice (Swarnalakshmi et al. 2013; Michalak et al. 2016; Hamed et al. 2018). Also, various microalgae species administered at optimised concentrations have been demonstrated to boost production of crop plants such as eggplant (Dias et al. 2016) , wheat (Swarnalakshmi et al. 2013; Michalak et al. 2016) and rice (Paudel et al. 2012; Priya et al. 2015). Recently, *Spirulina* based fertilisers have been proven to enhance growth in leafy vegetables such as Arugula (*Eruca sativa*), Bayam Red (*Ameranthus gangeticus*) and Pak Choy (*Brassica rapa* ssp.*chinensis*) (Wuang et al. 2016).

Microalgae positively stimulates many different processes for the benefit of the plant, such as nitrogen fixation, solubilisation of macro-and micronutrients, biocidial properties, photosynthesis, production of growth hormones, etc (summarised in Figure 1.3) (Renuka et al. 2018). Many scientists believe that microalgae could be a major player in the growing field of sustainable agriculture since they have many beneficial properties and are well established promoters of growth in plants (Win et al. 2018).

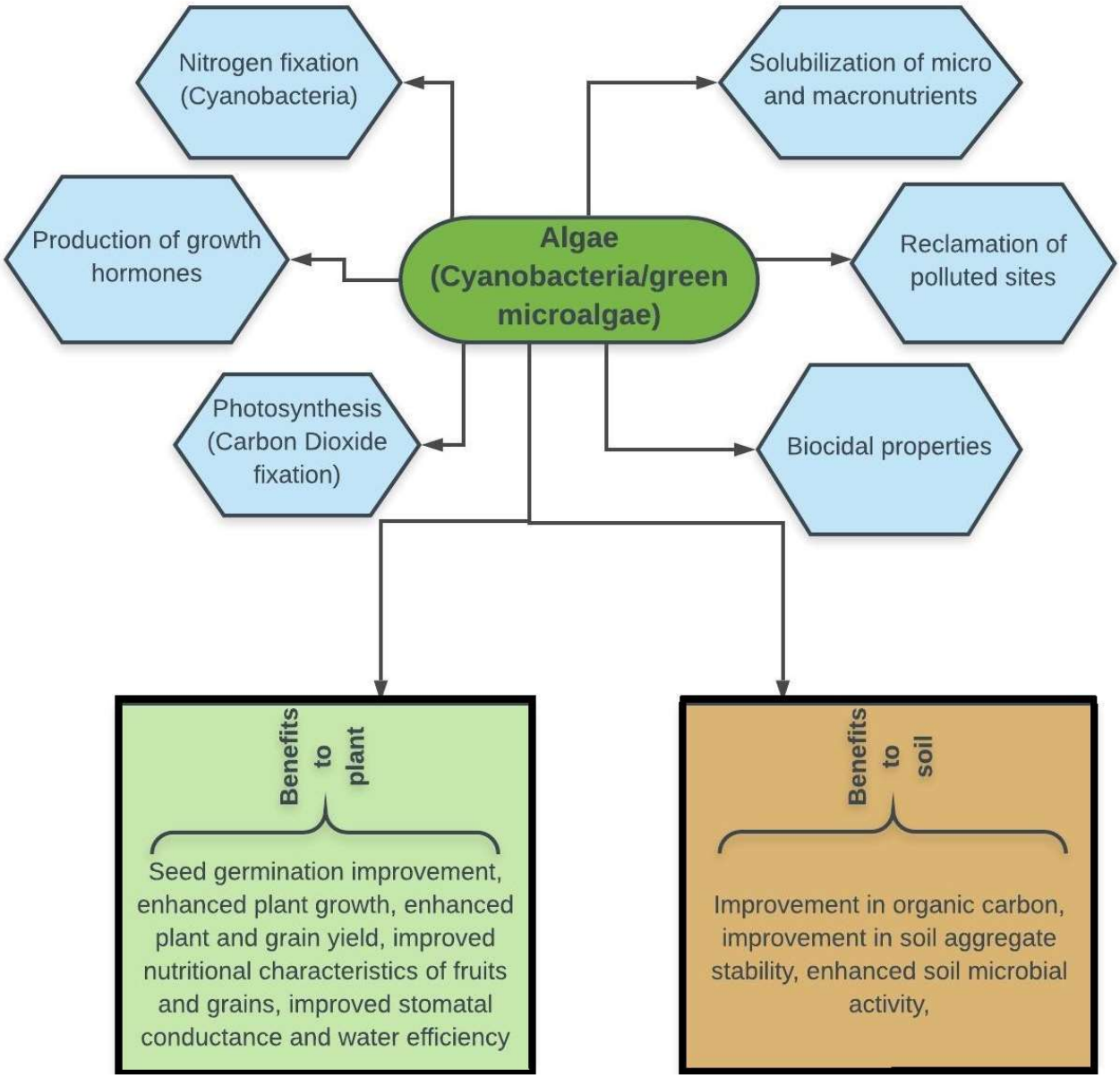


Figure 1.3: Overview of the beneficial effects which microalgae have on the plant and soil (adapted from Renuka et al., 2018).

1.8 Microalgae as a phytohormone producer

Microalgae have proved to not only promote growth in plants, but also act as pathogen resistance inducers. Cyanobacteria present in composts have been demonstrated to promote resistance towards pathogens in tomato, cotton and zucchini (Dukare et al. 2011; Babu et al. 2015; Roberti et al. 2015). Pathogen resistance has also been demonstrated in plants by applying microalgae directly to the soil (Kulik 1995; Biondi et al. 2004; Prasanna et al. 2013; Babu et al. 2015; Prasanna et al. 2015).

Various studies have been carried to further investigate how microalgae confer this resistance to the plants, where they have extracted various micro-algal compounds and how these extracts, in some cases, hinder directly the growth of different pathogens (Hellio et al. 2000; Kim, Kim 2008; Paulert et al. 2009; Jaulneau et al. 2010; Stadnik, Freitas 2014). There has been however no clear universal consensus on which hormonal or biochemical pathways the different microalgae extracts are hindering.

In one study, the plant pathogen response was shown to be promoted by ulvans, which are heteropolysaccharides from green algae of genus *Ulva*, and this induced pathogen response was shown to be dependent on JA signalling (Jaulneau 2010). Another study proposed that microalgae produce compounds which mimic those of pathogens, which then "tricks" the plant into thinking that it is infected, hence starting the defence responses (Stadnik, Freitas 2014). Another theory proposes that microalgae provide extra nutrients to the soil, making the plant more "fit", becoming easier for the plant to prevent pathogen attack (Babu et al. 2015). Overall, it has been described that both polysaccharides and phytohormones promote pathogen resistance in plants (Burketova et al. 2015; Singh et al. 2017).

Algae naturally produce phytohormones, namely CKs, in a broad range of profiles and levels (Jirásková et al. 2009; Hussain et al. 2010; Stirk et al. 2013; Žižková et al. 2016; Renuka et al. 2018). However, no connections have been made, so far, between the phytohormone production in microalgae and plant resistance promotion.

Chlamydomonas reinhardtii is a microalgae which naturally produces a large amount of CKs (Tian et al. 2006; Park et al. 2013). Hence, it would not be farfetched to assume that this alga may provide pathogen resistance to plants. Further investigation using knockout mutants in the CK biosynthesis pathway of these algae would probably prove CK's role in the induced pathogen defence responses of plants.

2. Aim of the work

This project aimed to characterize the cytokinin (CK) mediated biocontrol activity of *Chlamydomonas reinhardtii* (Chl) against *Pseudomonas syringae* pv. *tabaci* (PstT) infections in *Nicotiana tabacum* (*N. tabacum* or NT).
It also aimed to analyse the potential of Chl as a bio-stimulant in various crop species in order to combine its biocontrol activity with other beneficial properties to pave the way for making environmentally safe biocontrol agents.

In this sense, three hypotheses emerged that were addressed in this project:

- Can CK producing microalgae increase plant pathogen resistance?
- Is the inter-organism interaction mediated by phytohormones (CK)?
- Can microalgae have a bio-stimulating effect on cultivated species?

In order to validate our hypothesis, six mutants and two wild-type strains of Chl were used. The Chlamydomonas Library Project (CLiP) is an online collection of Chl mutants. From this collection two mutant strains related with CK-deficiency were purchased and used in the experiments. To bring the project closer to characterization of the underlying mechanism of CK effect, newly developed CRISPR/Cas9 Chl mutants were also used along with the already mentioned online ordered mutants from the CLiP project.

The mutants affected in CK biosynthesis were used to functionally address the role of CKs in a causal – analytical way: this is a very important part of the study since it moves the studies from correlative observations to a functional level, allowing much stronger conclusions on the role of CK production by the algae and the resistance mechanism.
A combination of various experiments which use either mutant Chls or wild types or both, helped us to bring closer to understanding the mechanisms of how this microalga foster nutrition as well as bio-protection to plants (Figure 2.1).

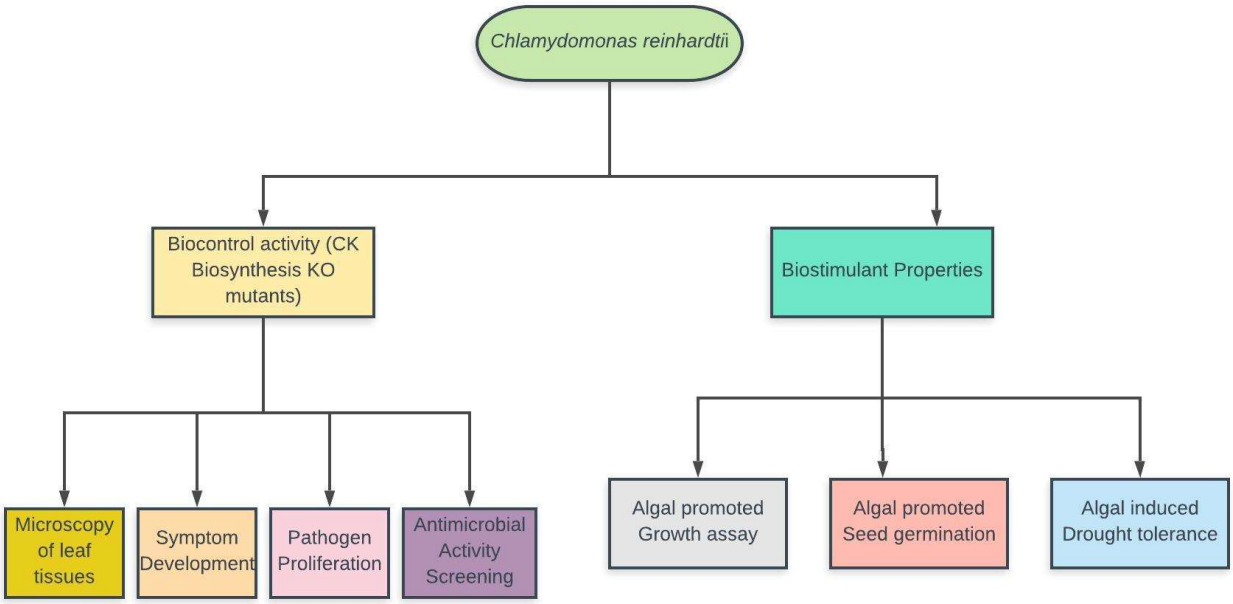


Figure 2.1: Overall plans of the various experiments performed in this project to explore the CK-dependent biocontrol properties as well as the bio-stimulant properties of *Chlamydomonas reinhardtii*.

3. Materials and Methods

3.1 *Chlamydomonas reinhardtii* strains

Both wild type and mutant strains (Table 3.1) of *Chlamydomonas reinhardtii* (Chl) were used in the various experiments depending on the objective. Two types of mutant strains were used –

i) **CLiP mutants**: Mutants ordered online from the Chlamydomonas Library Project (Li et al. 2016; Li et al. 2019).

ii) **CRISPR/Cas9 mutants**: Custom-made mutants from a collaborating laboratory in Germany using the CRISPR/Cas9 gene editing technology. All the CRISPR/Cas9 mutants used were clones of each other. CRISPR/Cas9 is a new revolutionary method which allows for precise targeted genome editing, resulting in well-defined mutants by utilizing the CRISPR-associated protein-9 nuclease of the *Streptococcus pyogenes* bacteria (Ran et al. 2013).

Table 3.1: List of wild type and mutant strains *Chlamydomonas reinhardtii* used in the experiments

Name	Type	Abbreviation	Description
Cre CC-5325	Wild type	CC 5325	High CK producing Wild type strain
Cre CC-125	Wild type	CC 125	High CK producing Wild type strain
LMJ.RY0402.045820	CLiP KO mutant	HMBPP / 045820	CC-5325 derived CK mutant where 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate reductase (responsible for formation of CK precursor) is knocked out
LMJ.RY0402.200648	CLiP KO mutant	cLOG / 200648	CC-5325 derived CK mutant where a portion of the coding region of the Lonely guy gene (responsible for CK synthesis activation) is knocked out
PH 144 2-LOG (Cre07.g340900)	CRISPR/Cas9 KO mutant	PH 144	CC-125 derived CK mutant where Lonely guy gene (responsible for CK synthesis activation) is knocked out. The mutation is in portion Cre07.g340900 of the gene.

PH 145 2-LOG (Cre07.g340900)	CRISPR/Cas9 KO mutant	PH 145	CC-125 derived CK mutant where Lonely guy gene (responsible for CK synthesis activation) is knocked out. The mutation is in portion Cre07.g340900 of the gene.
PH 146 2-LOG (Cre07.g340900)	CRISPR/Cas9 KO mutant	PH 146	CC-125 derived CK mutant where Lonely guy gene (responsible for CK synthesis activation) is knocked out. The mutation is in portion Cre07.g340900 of the gene.
PH 147 2-LOG (Cre07.g340900)	CRISPR/Cas9 KO mutant	PH 147	CC-125 derived CK mutant where Lonely guy gene (responsible for CK synthesis activation) is knocked out. The mutation is in portion Cre07.g340900 of the gene.

3.2 Chl culturing and maintenance

The microalgae strains were grown in 150 mL liquid HAP medium (Table 3.2) in Erlenmeyer flasks, spinning at 120 RPM at 23°C at 12h/12h day/night cycle. Cultures were kept as stock and experimental cultures. All the microalgae used in these experiments were taken from these Erlenmeyer flasks. Microalgae cultures used in experiments were inoculated every two weeks prior to experimental use. Inoculation was performed by transferring 1 mL of liquid stock culture to experimental flasks. Also, Chl cultures were maintained in solid HAP medium for backup and in case of contamination were initially plated on HAP plates with 10 µg/mL nalidixic acid (in 0.4M NaOH) and 40 µg/mL carbendazim (in water) to remove bacterial and fungal contamination (Mahan et al., 2005; Wang et al., 2016).

Table 3.2: Composition of HAP medium

Reagent	**Composition for 1 L**
TAP salts (Table 3)	25.00 ml
0.5 M HEPES	10.00 ml
1.0 M Na acetate	10.00 ml
Phosphate solution (Table 4)	0.375 mL

Hutner's trace (Table 5)	1.00 mL
Agar (for solid media)	15.00 g
H2O	up to 1 L

Stock Solutions

Table 3.3: Composition of TAP salts

Reagent	Molecular Weight [g/Mol]	For 1 L [g]
NH_4Cl	53.49	15
$MgSO_4.7H_2O$	246.47	4
$CaCl_2.2H_2O$	147.02	2
H_2O		Up to 1 L

Table 3.4: Composition of Phosphate solution

Reagent	Molecular Weight [g/Mol]	For 1 L [g]
K_2HPO_4	174.18	280.8
$MgSO_4.7H_2O$	136.09	140.4
H_2O		Up to 1 L

Table 3.5: Composition of Hutner's Trace Elements

Reagent	Molecular Weight	For 100 mL [g]
EDTA, disodium salt	372.2	5.0
H_3BO_3	61.83	0.145
$ZnSO_4.7H_2O$	287.54	0.01
$MnCl_2.4H_2O$	197.9	0.09
$FeSO_4.7H_2O$	278.01	0.499
$CoCl_2.6H_2O$	237.95	0.161
$CuSO_4.5H_2O$	249.68	0.157
$(NH_4)_6Mo7O_{24}.4H_2O$	1235.86	0.11
H_2O		Up to 1 L

3.3 *Pseudomonas syringae* pv. *tabaci* culturing and maintenance

Pseudomonas syringae pv. *tabaci* (PstT) stock culture was grown on LB medium plates (Table 3.6) with tetracycline (20 mg/L) at 28ºC for 24h (or 48h if in some cases there was less growth). After that they were stored at 4°C. Fresh cultures were made every other week by re-plating. LB medium was autoclaved over 2 hours and then brought down to 60°C before the antibiotic addition.

Table 3.6: Composition of LB medium

Reagent	**For 1 L [g]**
Yeast Extract	5.0
Sodium Chloride (NaCl)	10.0
Tryptone	10.0
Agar – agar (Only for solid medium)	15.0
Tetracycline	0.02
Demineralised water (H_2O)	Upto 1 L

3.4 Planting of *Nicotiana tabacum*

Nicotiana tabacum (NT) seeds were put in 15 cm pot with soil soaked in a fungus-gnat controlling agent (Gnatrol SC 30 mL/5L) and water, in a cold room for 3 days, before being put in a growth chamber at 13°C. 1-2 weeks after germination, the seedlings were then replanted into a 12 cm pot soaked in fungus-gnat controlling agent and water, and put in the greenhouse (at 20°C to 24°C and 16 h/8 h day/night cycle). After 2 weeks of growth in 16h/8h day/night cycle, the plants were replanted into bigger 15 cm pots soaked in a fungus gnat controlling agent and water. A distance has been maintained between the tobacco plants and yellow sticky traps were installed to control pests (e.g. white flies).

3.5 Chl solutions preparation

In most experiments, both live microalgae and microalgae extracts were used. This was to test whether the mechanism behind the biocontrol was connected to the algae being alive or if extracts were more efficient as is makes the contents inside the microalgae cells more available.

Extracts were made on the same day as the plants were inoculated with microalgae. In some experiments, microalgae culture was also used.

Microalgae live suspension (also called microalgae suspension): Microalgae were transferred to a 50 mL falcon tube and centrifuged for 30 min at 20°C. **Supernatant** was discarded for some experiments and saved in a separate tube for the seed germination experiments. Then, the pellet was weighed. Demineralized autoclaved water was added to the algae pellet to obtain a suspension of 75 g algae/L water.

Microalgae Extract: For extractions, an aliquot of the microalgae solution was placed in a 100 mL beaker and microwaved at 1000 W for 1 min and subsequently 270 W for 4 min (McMillan 2013). The extract was then transferred into a 50 mL falcon tube and cooled down to room temperature before further use.

Microalgae culture: Microalgae were grown in liquid HAP media for two weeks prior to their use, and the microalgae were taken directly from Erlenmeyer flasks and used in experiments without any treatments.

3.6 Preparation of PstT and infiltration

With a pipette tip, PstT obtained from 1-week old main stock was transferred to 4-5 mL LB-medium with tetracycline. This culture was then incubated at 28°C overnight shaking at 110 RPM. Approximately 1 mL of the overnight culture was transferred to 50 mL LB medium, incubated at 28°C, and harvested at OD of 0.4-0.8.
The culture was transferred into 50 mL falcon tube and centrifuged at 3500 RPM at 4°C for 10 min. The supernatant was discarded, and the pellet was re-suspended and diluted in 10 mM $MgCl_2$ to OD of 0.2. The cell suspension was infiltrated on 6-8 weeks old NT plants using a needleless syringe in the abaxial side of the leaf.

3.7 Infiltration experiments on NT for symptom development

To characterize the symptom development caused by PstT, infiltration experiments were performed. The time from which the leaves were primed with Chl was considered as 0h experimental time, and the development of symptom on the leaves was scored at 72h, 96h and 168h experimental time. Infiltration experiments on NT were performed with Chl strains PH 144, PH 145, PH 146, PH 147, 200648 and 045820, along with their respective wild type Chl strains (CC 125 and CC 5325). There were two repetitions of the experiments with the CRISPR/Cas9 mutants, while the experiments with the CLiP mutants were done only once.

In an infiltration experiment, 5 plants were inoculated with two different Chl strains as a pre-treatment prior to pathogen infiltration. On each plant, two leaves were pre-treated, each with one of the two Chl WT strains. In addition, each microalgae strain was applied as twice, one in the form of suspension and in the other has extract. Mock treatment (water infiltration) was included as a negative control.

Leaves were divided into three areas (Figure 3.1):

• Mock solution (left to midrib)

• Chl Suspension (upper right to midrib)

• Chl Extract (lower right to midrib)

These areas were located between the leaf veins on each side of the midrib. Each of these areas were sub-divided into 3 sections.

• Pathogen-only (section closest to the midrib)

• Pathogen and pre-treatment overlapping (Mid-section)

• Pre-treatment-only (section closest to the leaf edge)

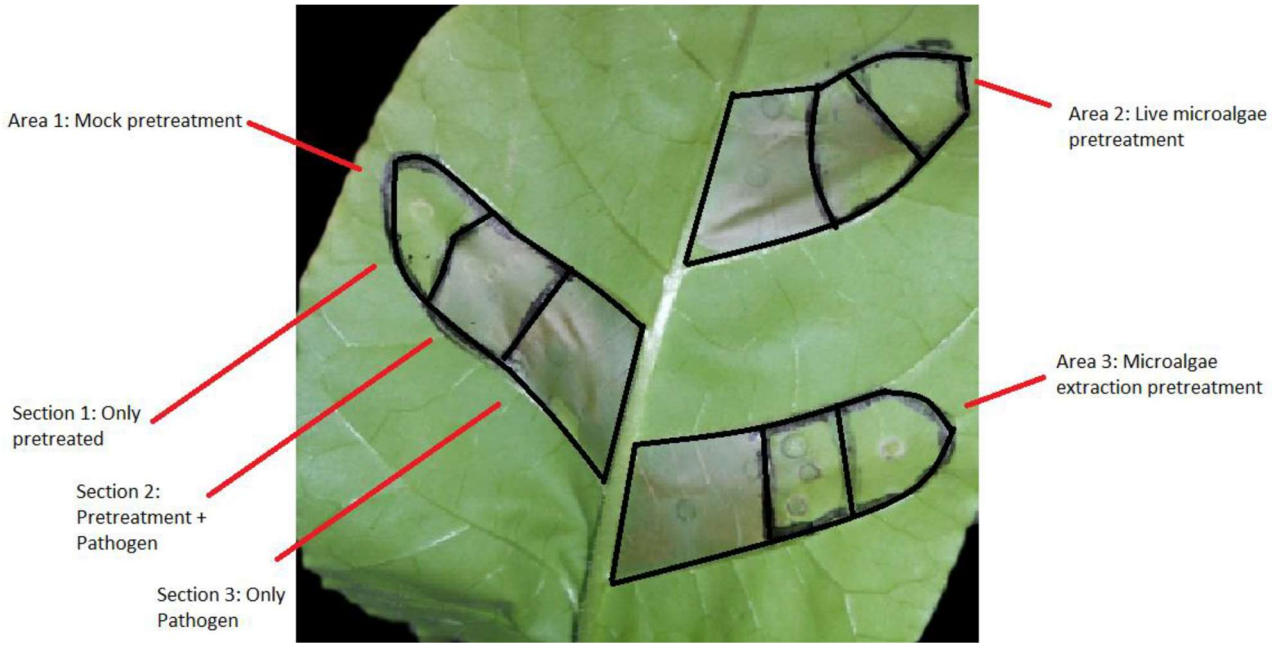


Figure 3.1: Experimental setup of pathogen symptom development determination.

At 48h experimental time the leaves were infiltrated with PstT as mentioned above. The leaves were photographed every 24h hours subsequently to the infiltration, for 4 days. The symptoms were then evaluated into symptom scores where each overlapping section was scored from 0 to 5. The score was given in relation to the percentage of observed necrosis on leaves. No symptoms were scored as 0, while 100 % necrosis was scored as 5 (Table 3.7).

Table 3.7: Classification of *P. syringae* infection symptoms in *N. tabacum*. The percentages of symptom development correspond to the symptoms as seen inside the black circles.

Category (score)	**0**	**0.5**	**1**	**2**	**3**	**4**	**5**
Symptoms	No symptoms	≤ 75 % Chlorosis	> 75 % Chlorosis	≤ 10 % Necrosis	11-50 % Necrosis	51-75 % Necrosis	76-100 % Necrosis
Symptoms in *N. tabacum*							

3.8 Bacterial quantification experiment

Leaves were infiltrated with microalgae strains, and after 48 hours they were infiltrated with PstT in the same manner has described in the previous section. Leaves across 5 plants were divided into 5 areas:

- Two areas for microalgae suspension,
- Two areas for microalgae extracts
- One area for mock treatment.

From each area, 0.5 mm leaf discs were collected in Microtube tubes every 24h, for 3 days post infection with PstT. The discs were grounded in 200 µL of 10 mM $MgCl_2$ using a sterilized pistil and filled to 1 mL. Afterwards, a 10-fold dilution was made by transferring 100 µL to another Eppendorf tube with 900 µL of 10mM $MgCl_2$. Both the original solution and the diluted samples were plated by spotting triplets of 10 µL drops on LB medium with tetracycline. The plates were incubated for 24h at 28°C and the microcolonies were counted using a dissecting microscope to observe the pathogen proliferation.

The 0h experimental time corresponds to the time were leaves were primed with Chl and for the purpose of bacterial count, the leaves were scored at 48h, 72h and 96h experimental time in order to track the growth of bacteria over 3 days in the presence of the different treatments.

Pathogen proliferation experiments were done with Chl strains PH 144, PH 145, PH 146, PH 147, 200648 and 045820, and with their respective wild type Chl strains (CC 125 and CC 5325). There were two repetitions of the experiments with the CRISPR/Cas9 mutants, while the experiments with the CLiP mutants were done only once. Each experiment had five biological replicates.

3.9 Antimicrobial activity

In order to determine the antimicrobial activity of tobacco leaves primed with several Chl strains three different methods were used:

i) **Sodium salt and EDTA based extraction (Abdelmohsen et al. 2011)** (Figure 3.2): The strains tested with this method were CC 125, PH 144, PH 145, PH 146 and PH 147. Leaves from five *N. tabacum* plants were pre-treated with two microalgal strains, with both suspension and extraction inoculates. Every day, for five days subsequently to the pre-

treatment, plants leaves were harvested, to create a 5-day overview of the antimicrobial activity. During the harvest, the leaf portions were put into Microtube tubes and dropped into liquid nitrogen. The frozen leaves were grounded, and 0.5 g were re-suspended in 1 mL buffer A (10 mM Na_2HPO_4, 15mM NaH_2PO_4, pH =7) and then stirred for 1 h at 4°C. Afterwards, they were centrifuged at 20°C, 3500 RPM for 20 min. Supernatants were used as intracellular fraction (extract 1). Pellets were re-suspended in 1 mL buffer B (10 mM Na_2HPO_4, 15mM NaH_2PO_4, pH = 7, 1 M NaCl, 15mM NaEDTA) and stirred overnight at 4°C. Samples were centrifuged again at 20°C, 3500 RPM. The supernatants were dialyzed overnight against demineralized water at 4°C. The following day the dialyzed fraction (extract 2) was collected in 2 mL Microtube tubes. Extract 1 and 2 were then pooled and then used in an agar diffusion test. One mL of PstT was distributed at 0.2 OD to LB medium plates. The plates dried for 10 minutes and excess culture-suspension was removed. Plates were sectioned into six areas, two sterile filter paper discs - 0.5 mm thick and 5 mm diameter big - were placed in every section of the plate (Figure 3.3). On every paper disc, 10 µL of the pooled extracts were added. Plates were incubated at 28°C for 24 hours. The effect of the antimicrobial activity of the extract was determined by the size of the diameter of the halo. As a positive control, kinetin (a cytokinin) treated *N. tabacum* samples (Großkinsky et al. 2011) was used, while buffer A was used as a negative control.

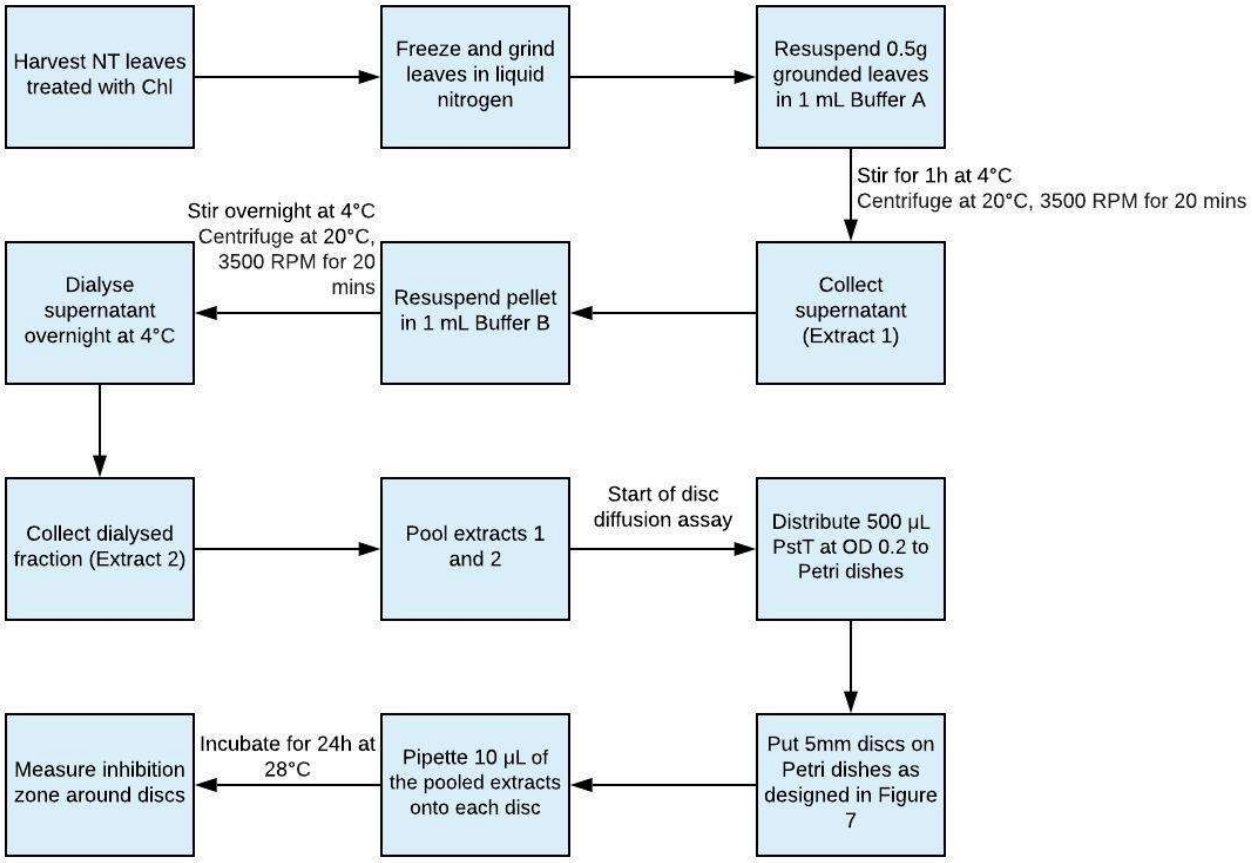


Figure 3.2: Scheme of the sodium salt and EDTA based extraction protocol for antimicrobial activity screening of plant extracts (Adapted from Abdelmohsen et al. 2011).

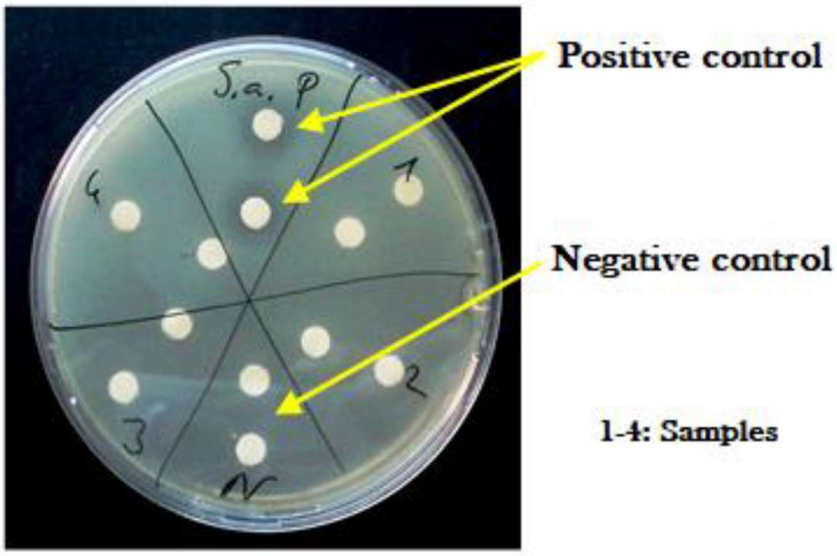


Figure 3.3: Experimental setup of antimicrobial activity determination for disc diffusion assays, P: Positive control, N: Negative control and 1-4 the samples in question.

ii) **Ethanol-based extraction (Biswas et al. 2013)** (Figure 3.4): Tobacco leaves were infiltrated with Chl solutions, as previously described. The used strains were CC 5325, CC 5325 + PstT, PstT, and water. After infiltration, the leaves were left for 2 days and then put in the oven at 70 ℃ to dry for 24h. Then, the dry leaves were weighed (around 0.05g each), and then they were ground with a sterilised pistil in Microtube tubes. They were subsequently dissolved in 1 mL 80% ethanol and then left to stir at room temperature for 48h. The samples were then centrifuged at 10000 RPM for 10 minutes. Then they were filtered through a syringe filter by passing through Chromafix column (> 45µM) and then left to dry in a SpeedVac overnight. The next day, the extracts were filled to 500 µL with 80% ethanol to be used in the antimicrobial assay experiments.

A) *Disc diffusion assay* – overnight PstT cultures with an OD 0.13 were spread evenly on the agar plates and left to dry. Then, sterile filter paper discs with a diameter of 6 mm were impregnated with testing solution (20 µL/disc), dried (3 times impregnation and drying) and placed on the agar plates inoculated with Pst. Chloramphenicol (1 mg/mL) was used as a positive control, and 80% ethanol was the negative control. There were 2 replicates of each plate. The plates were inverted and incubated for 24 h at 28℃. Inhibition zones around the discs were measured the next day.

B) *Broth microdilution assay* (Eloff 1998) – 50 µL PstT solution was put into the wells A1-A1, B1-B7, C1-C7 and D1-D7 in a 96-well microtitre plate. The wells A8-9, B8-9, C8-9 and D8-9 had no bacteria, but only 100 µL ethanol and only LB respectively. Then, the first 4 columns were treated with the 50 µL plant extracts respectively, followed by putting 50 µL of 1 mg/mL chloramphenicol, 80 % ethanol and only Pst. Then, the plate was wrapped in parafilm and left to incubate overnight at 28 ℃. The following day, the plate was taken out and then 50 µL INT (2 mg/mL) was put into each of the wells. INT is a tetrazolium salt based dye which changes colour from colourless to pink in the presence of respiratory activity. The deepness of the colour directly corresponds to the number of bacteria present in the solutions. The plate was then kept in the dark for 30 minutes and then colour change was observed.

iii) **Methanol-based extraction** (Parekh, Chanda 2007) (Figure 3.4): Entire tobacco leaves were infiltrated with algae solutions as previously described. The used strains were CC 125, CC 125 + PstT, PstT and water. Two days after infiltration, the leaves were harvested

and then grounded in liquid nitrogen and weighed. One gram of grounded leaves was taken from each treatment. One gram was put in a Microtube tube and dissolved in 2 mL 80% methanol and then left to stir at -4℃ for 24h. The samples were then centrifuged at 14000 RPM for 15 minutes. Afterwards samples were filtered through a syringe filter by passing through Chromafix column (>45 µM) and left to dry in a SpeedVac overnight. The next day, 500 µL of 80% methanol were added to the extracts and used in the antimicrobial assay experiments.

A) *Disc diffusion assay* – Overnight PstT cultures of OD 0.13 were spread evenly on the agar plates and left to dry. Then, sterile filter paper discs with a diameter of 6 mm were impregnated with testing solution (20 µL/disc), dried (3 times impregnation and drying) and placed on the agar plates inoculated with Pst. Chloramphenicol (0.25 mg/mL) was used as a positive control, and 80% methanol was the negative control. There were 2 replicates of a plate. The plates were inverted and incubated for 24 h at 28°C. Inhibition zones around the discs were measured the next day.

B) *Broth microdilution assay* (Eloff 1998) – 50 µL PstT solution was put into the wells A1-A1, B1-B7, C1-C7 and D1-D7 in a 96 well microplate. The wells A8-9, B8-9, C8-9 and D8-9 had no bacteria, but only 100 µL 80% methanol and only LB. Then, the first 4 columns were treated with the 50 µL plant extracts respectively, followed by putting 50 µL of 0.25 mg/mL chloramphenicol, 80 % methanol, and only Pst. Then, the plate was covered with aluminium foil and left to incubate overnight shaking at 28 ℃. The following day, the plate was taken out and then 50 µL INT (2 mg/mL) was put into each of the wells. INT is a tetrazolium salt-based dye which changes colour from colourless to pink in the presence of respiratory activity. The deepness of the colour directly corresponds to the number of bacteria present in the solutions. The plate was then kept in the dark for 30 minutes and then colour change was observed.

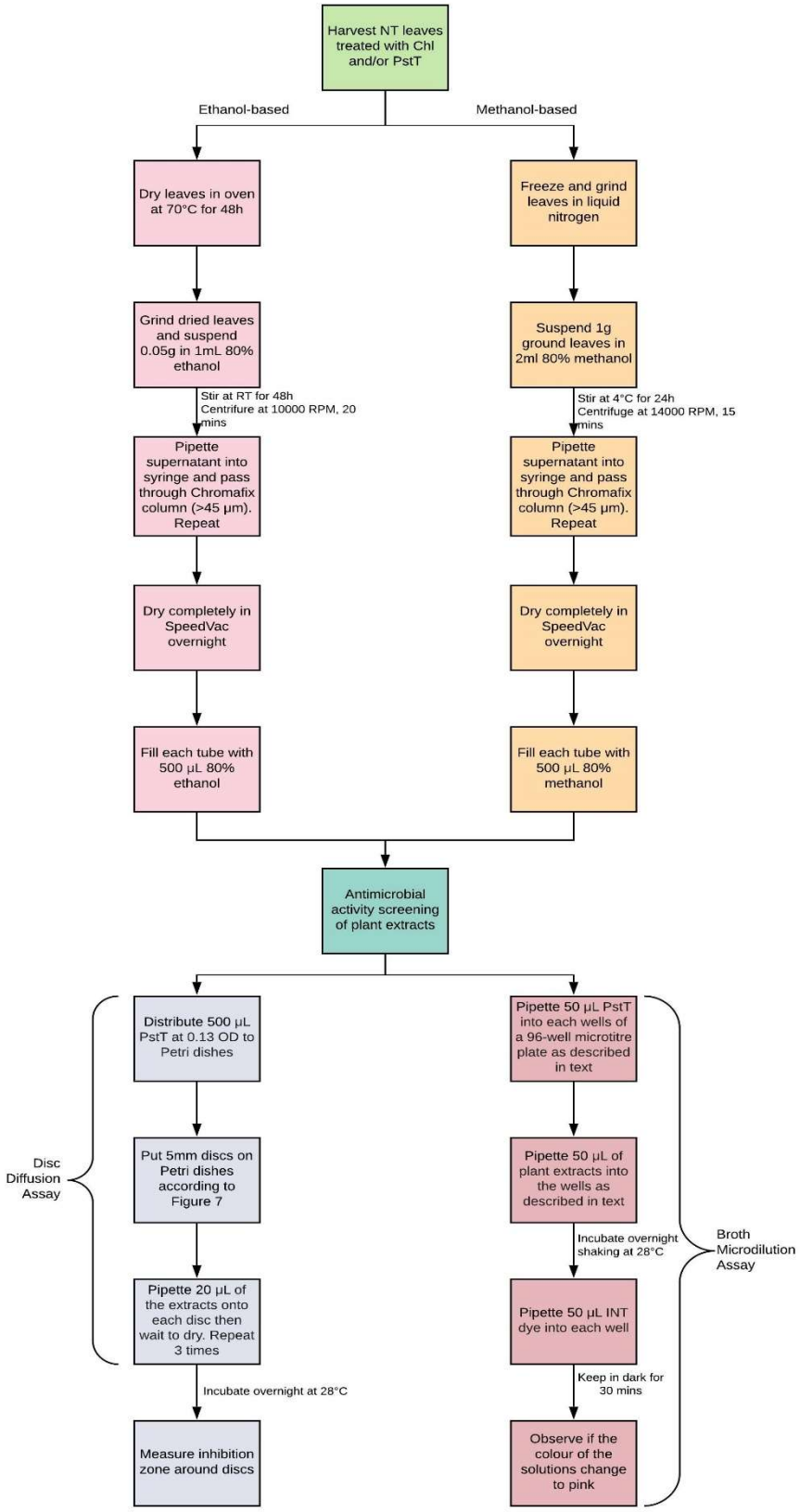


Figure 3.4: Scheme of ethanol and methanol-based extractions to detect the antimicrobial activity of plant extracts (Adapted from Eloff 1998; Parekh, Chanda 2007; Abdelmohsen et al. 2011; Biswas et al. 2013)

3.10 Optical Microscopy

Microscopy was carried out to compare and observe the differences PstT had between the wild type (CC 5325) treated leaves and mock treated leaves. Leaf tissue squares (10 x 10 mm) were cut with a razor from both the mock and CC 5325 treated samples up to four DPI and immediately stained with aniline blue in lactophenol (0.01g aniline blue, 5 mL lactic acid and 10 mL H_2O) (Whitelaw-Weckert et al. 2011). Then, the leaf portions were observed under a Leica DM 2000 microscope at 100x magnification.

3.11 Algal promoted *in vitro* tomato seed germination

The suspension, extract, culture and supernatant solutions of microalgae strains CC 125, PH 144, PH 147, CC 5325, 200648 and 045820 were used on tomato seeds (variety Moneymaker) for this algal promoted seed germination test. Chl suspensions were made from 2-week old cultures. The seeds were surface sterilized with 40 mL of 2.7% sodium hypochlorite for 15 minutes and rinsed thoroughly in demineralized water. Seeds were vernalized at 4°C for 48h. 500µL of the respective microalgae suspensions were pipetted onto the Petri dishes with 1% agar containing 0.5x MS. After the microalgae suspensions dried (10-15 minutes later), 15 seeds were placed on each Petri dish. There were two Petri dishes per microalgae treatment, each containing 15 seeds, so the total number of seeds for each treatment was 30. The plates were incubated at room temperature with 12h/12h day/night cycle. Seed germination was initially checked after 72h, and again every 24 hours (Ghaderiardakani et al. 2019). The seeds were counted to be germinated if at least 2 mm of the radical had emerged. Also, the germination percentage was calculated. Germination percentage is an estimate of the viability of a population of seeds and was calculated as

$$GP = (\text{number of germinated seeds}/\text{total number of seeds}) \times 100.$$

3.12 Algal promoted growth assay of tomato and NT

The microalgae solutions chosen to be tested for this experiment were CC 125 culture, CC 125 suspension, CC 125 extract and control (only water) to be tested on tomato and tobacco (Figure 3.5). Eight 10-packs consisting of ten wells were filled with sowing soil, four 10-packs for tomato and four

10-packs for tobacco. A pre-germinated seed was put into each well (7 days after sowing). And then after the addition of 5 mL of milli-Q water, 10 mL of the solutions of the different microalgae treatments were poured onto each of the seedlings in the wells and all the plants were incubated in the greenhouse for 2 weeks. At the end of the experiment, all plant parts were harvested and washed with care over a sieve and then weighed, then differences between their weights were analysed (Kholssi et al. 2019).

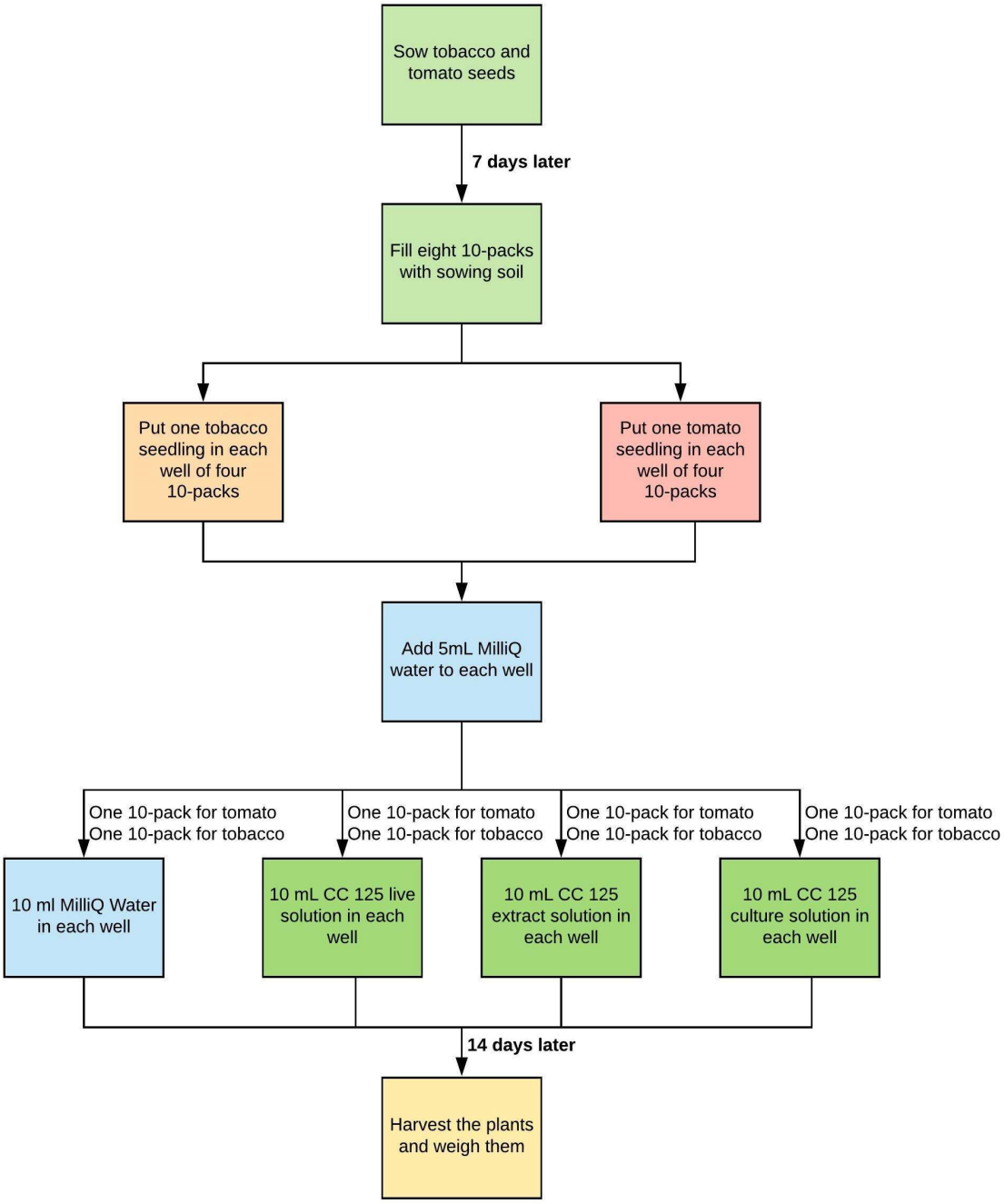


Figure 3.5: Scheme of the algal promoted growth assay of tobacco and tomato (Adapted from Kholssi et al. 2019).

3.13 Drought tolerance experiment in *Arabidopsis*

The microalgae treatments used for this experiment were CC 125 culture, CC 125 suspension, CC 125 extract and control (only water). Thirty-six wells for each treatment were filled with soil to the brim (6 wells in one 2x3 six-pack). Then, they were watered, saturated and weighed to get around 250 g per six-pack. Then 2-3 *Arabidopsis* seeds were sown in each well. Afterwards, the trays filled with six-packs were covered with plastic bags and left to vernalise at 4°C for 5-7 days. The trays were then moved to the growth chamber or greenhouse and left to grow for 10 days. In the meanwhile, plants were watered every 2 days. As already mentioned, after 10 days, seedling selection was done so that each well had similar sized seedlings. The drought tolerance experiment was then started (Bresson 2013). A day later, 2 mL of the respective microalgae treatment solutions was poured on top of each of the seedlings. Plants were given 2 days to adjust to the new treatments. The drought condition was then started by stop watering plants until the day before harvesting. Control plants were watered every two days. Seven days after microalgae inoculation, each six-pack was weighed again, and the well-watered plants were harvested and individually weighed. The drought subjected plants were re-watered and then harvested and individually weighed the following day. Then the soil water content (SWC) was measured for all the plants:

$$SWC = \frac{(soil\ weight\ at\ harvest - fully\ dried\ soil\ weight)}{Fully\ dried\ soil\ weight} * 100$$

4. Results

4.1 Priming with microalgae decreased symptoms caused by PstT in NT

NT leaves were screened for PstT induced symptoms in several individual experiments where the symptoms of the WT Chl strains were compared with various Chl KOs. These experiments were done in order to visualise the effect that microalgae had on the plants in terms of protecting them against PstT infection, and if this protection differed between the WT and the mutant strains.

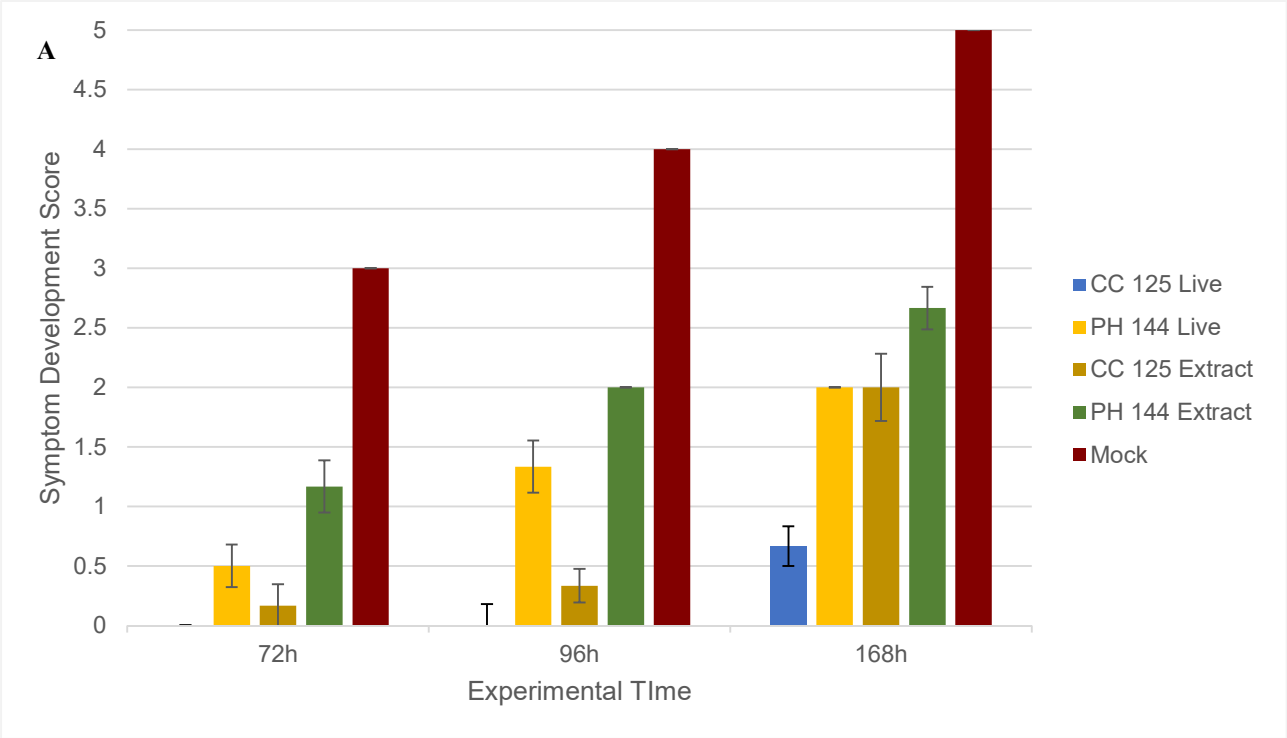
4.1.1 WT CC-125 versus 2-LOG KOs

The 2-LOG KO Chl mutants were generated by knocking out a portion of the Lonely Guy gene (LOG). Several clones of this mutant (PH 144, PH 145, PH 146 and PH 147) were used to confirm if the LOG gene is indeed an integral part of the cytokinin biosynthesis pathway or not. In addition, using several mutants offers the advantage to have more reliable results.

The infiltration experiments were performed to determine whether the LOG gene (indirectly CK) had any effect on the development of PstT-induced symptoms compared to the WT CC 125 strain and water (mock). The assays were repeated twice for each mutant strain, each having 5 biological replicates.

In the first experiment the development of PstT-induced symptoms in NT leaves treated with PH 144 were compared against CC 125 and mock. In this assay we observed that symptoms in leaves treated with PH 144 (suspension and extract) were significantly more severe when compared to leaves treated with CC 125 (suspension and extract). Nevertheless, PH 144 mutant still gave some additional protection to the plant when compared with the mock treated patch of leaves (Figure 4.1A).

In the replica we observed similar results where the suspension of PH 144 showed significantly more severe symptoms on NT leaves than the CC 125, but there were no significant differences between the effects of the CC 125 and PH 144 extract solutions. Nevertheless, PH 144 mutant still gave some additional protection to the plant when compared with the mock treated patch of leaves (Figure 4.1B).



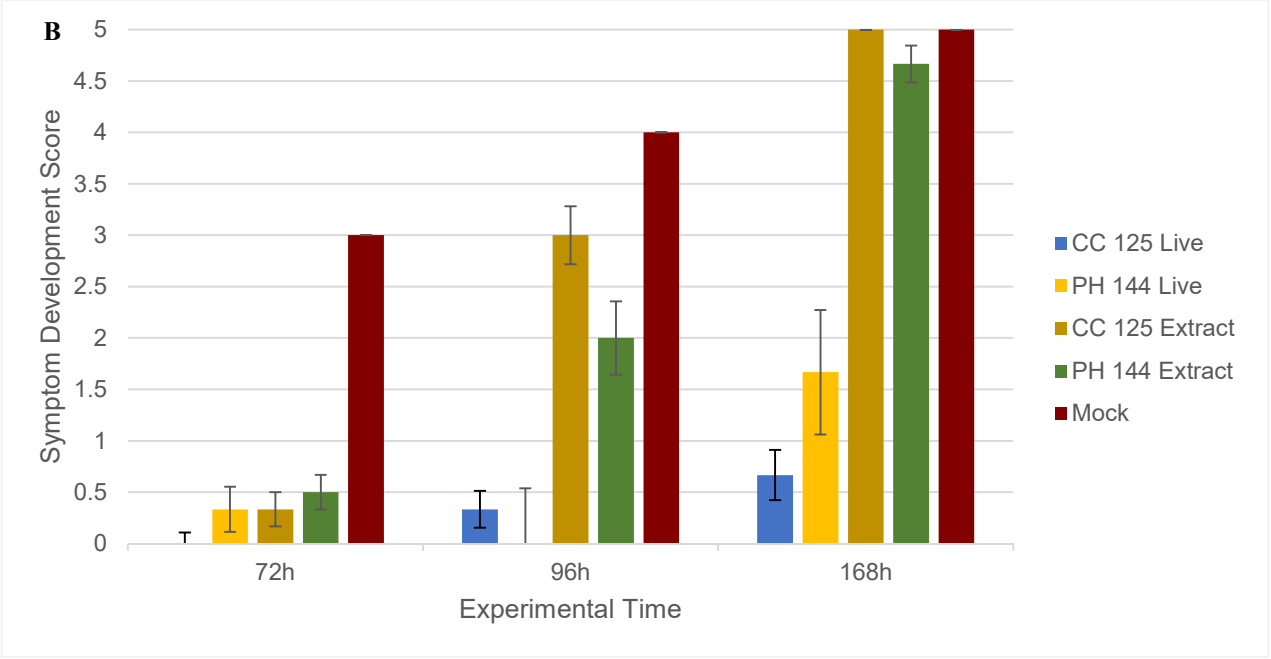


Figure 4.1 Symptoms observed in the NT leaves at 72h, 96h and 168h of experimental time by scoring the patch of the leaf which was primed with the respective Chl strains CC 125 and PH 144 first, and then infiltrated with PstT at 48h. A. Experiment 1; B. Experiment 2. The mock, CC 125 suspension and PH 144 suspension treated portions are significantly different from each other, with the CC 125 suspension showing less symptoms (Unpaired Student's t-test, $p << 0.05$, n=5).

The symptoms induced by PstT in infiltrated leaves with CC 125 versus PH 144 were easily distinguishable from 72h to 168h of experimental time. The patches with CC 125 suspension and CC 125 extract were greener than the ones with PH 144 suspension and extract, while the mock treated patches had undergone complete necrosis by 168h (Figure 4.2).

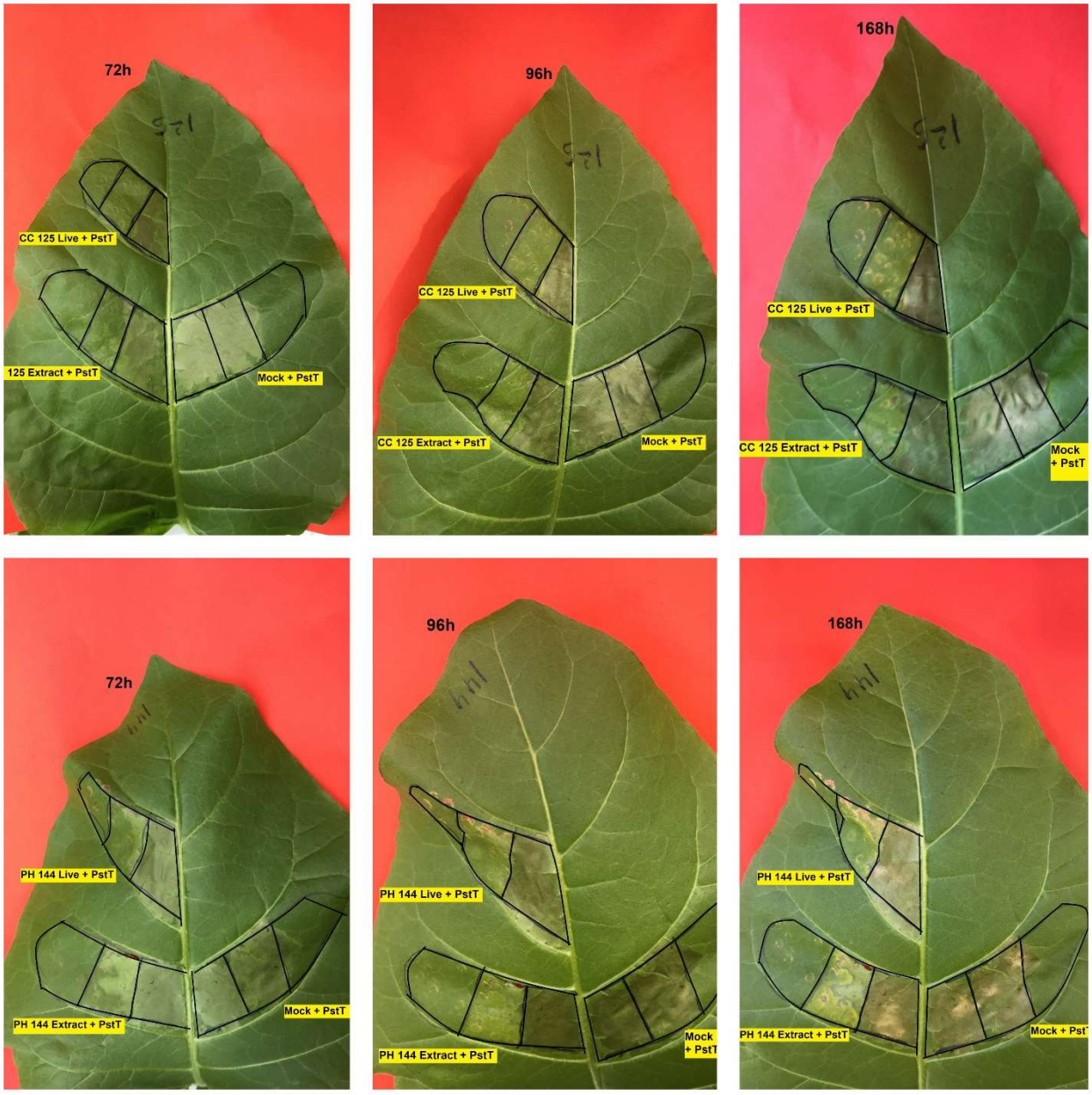


Figure 4.2: Examples of symptoms caused by PstT in NT leaves treated with WT CC 125 (above) and mutant PH 144 (below) at 72h, 96h and 168h experimental time.

The PstT symptom development in NT leaves was also compared between PH 145 mutant strain, WT CC 125 strain and mock in two sets of experiments. In the first experiment the development of PstT-induced symptoms in NT leaves treated with PH 145 were compared against CC 125 and mock. In

this assay we observed that symptoms in leaves treated with PH 145 (suspension and extract) were significantly more severe when compared to leaves treated with CC 125 (suspension and extract). Nevertheless, PH 145 mutant still gave some additional protection to the plant when compared with the mock treated patch of leaves (Figure 4.3A).

The second experiment with CC 125 versus PH 145 turned out to be comparatively less successful than the first one, since the protection offered by CC 125 suspension and PH 145 suspension were not significantly different from each other. However, there was a significant difference between the effects on the CC 125 extract and PH 145 extract treated portions, with the later exhibiting more severe symptoms. Nevertheless, the PH 145 mutant still provided additional protection to the plant when compared to the mock treated patch of leaves (Figure 4.3B).

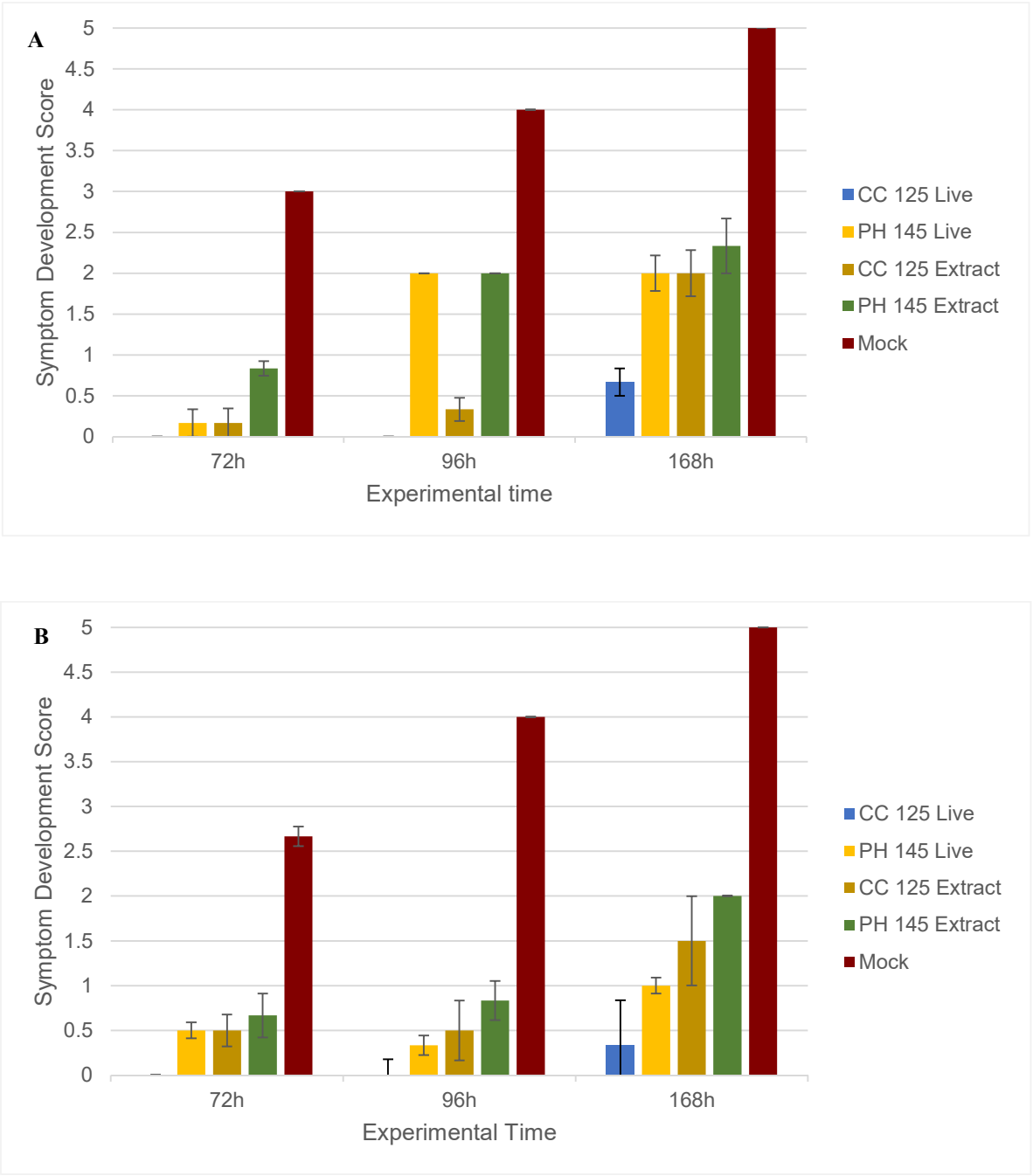


Figure 4.3: The symptoms observed in the NT leaves at 72h,96h and 168h of experimental time by scoring the patch of the leaf which was primed with the respective Chl strains CC 125 and PH 145 first, and then infiltrated with PstT. A. experiment 1; B. experiment 2. The CC 125 suspension and PH 145 suspension treated portions were not significantly different from each other, (Unpaired Student's t-test, $p > 0.05$, n=5). However, the CC 125 extract and PH 145 extract portions were significantly different from each, with the CC 125 treated portions showing less symptoms (Unpaired Student's t-test, $p << 0.05$, n=5).

The symptoms induced by PstT in infiltrated leaves with CC 125 versus PH 145 were easily distinguishable from 72h to 168h of experimental time. The patches with CC 125 live and CC 125 extract were greener than the ones with PH 145 suspension and extract, while the mock treated patches had undergone complete necrosis by 168h (Figure 4.4).

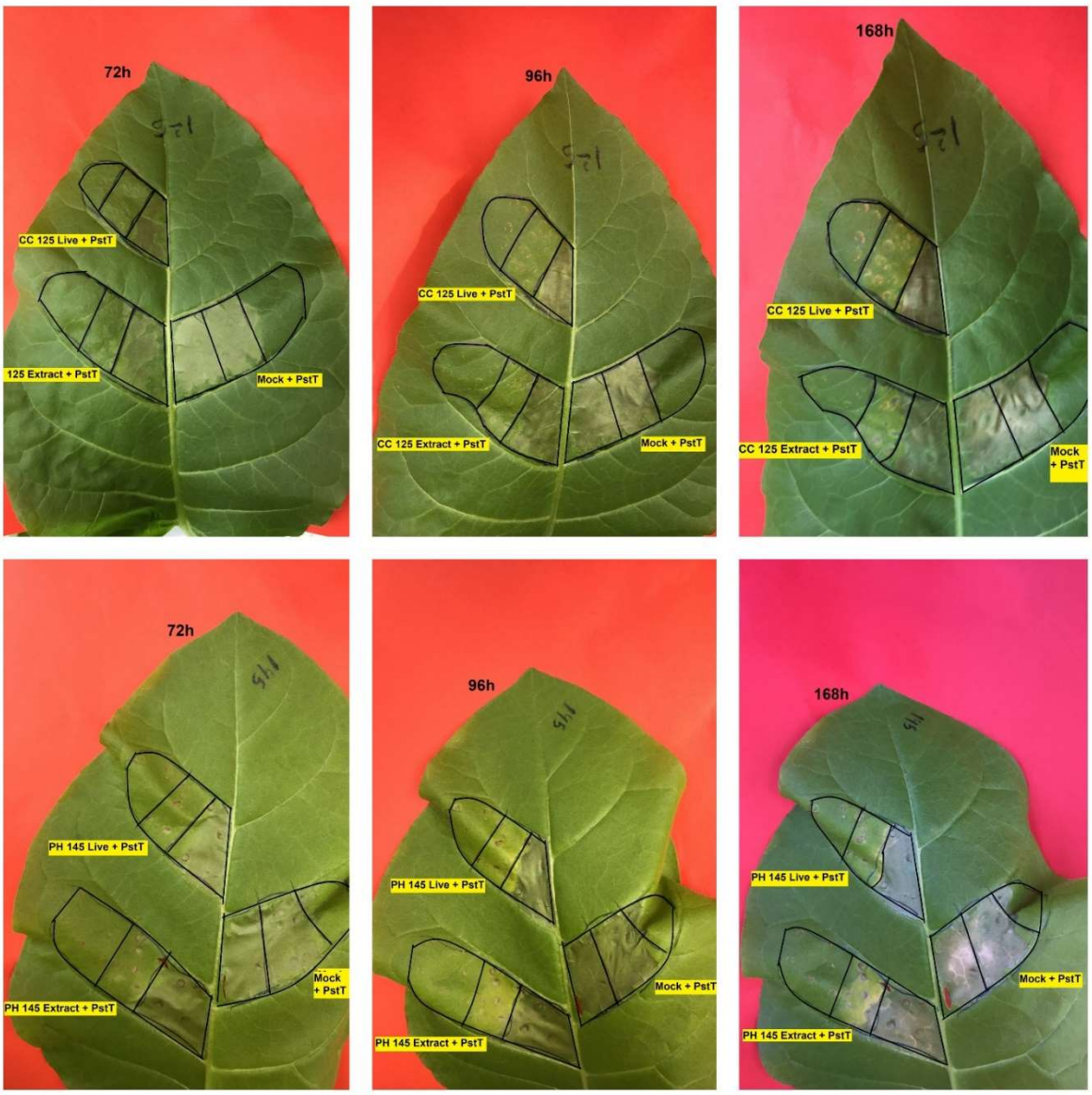


Figure 4.4: Examples of symptoms caused by PstT observed in NT leaves treated with WT CC 125 (above) and mutant PH 145 (below) at 72h, 96h and 168h experimental time.

In both experiments performed with CC 125 versus PH 146, the symptom development of PH 146 was compared against CC 125 and mock. In this assay we observed that both the suspension and the extract from PH 146 and CC 125 treated portions were not significantly different from each other and that the PH 146 mutant offered similar additional protection as the WT to the plant when compared with the mock treated patch of leaves (Figure 4.5A,4.5B).

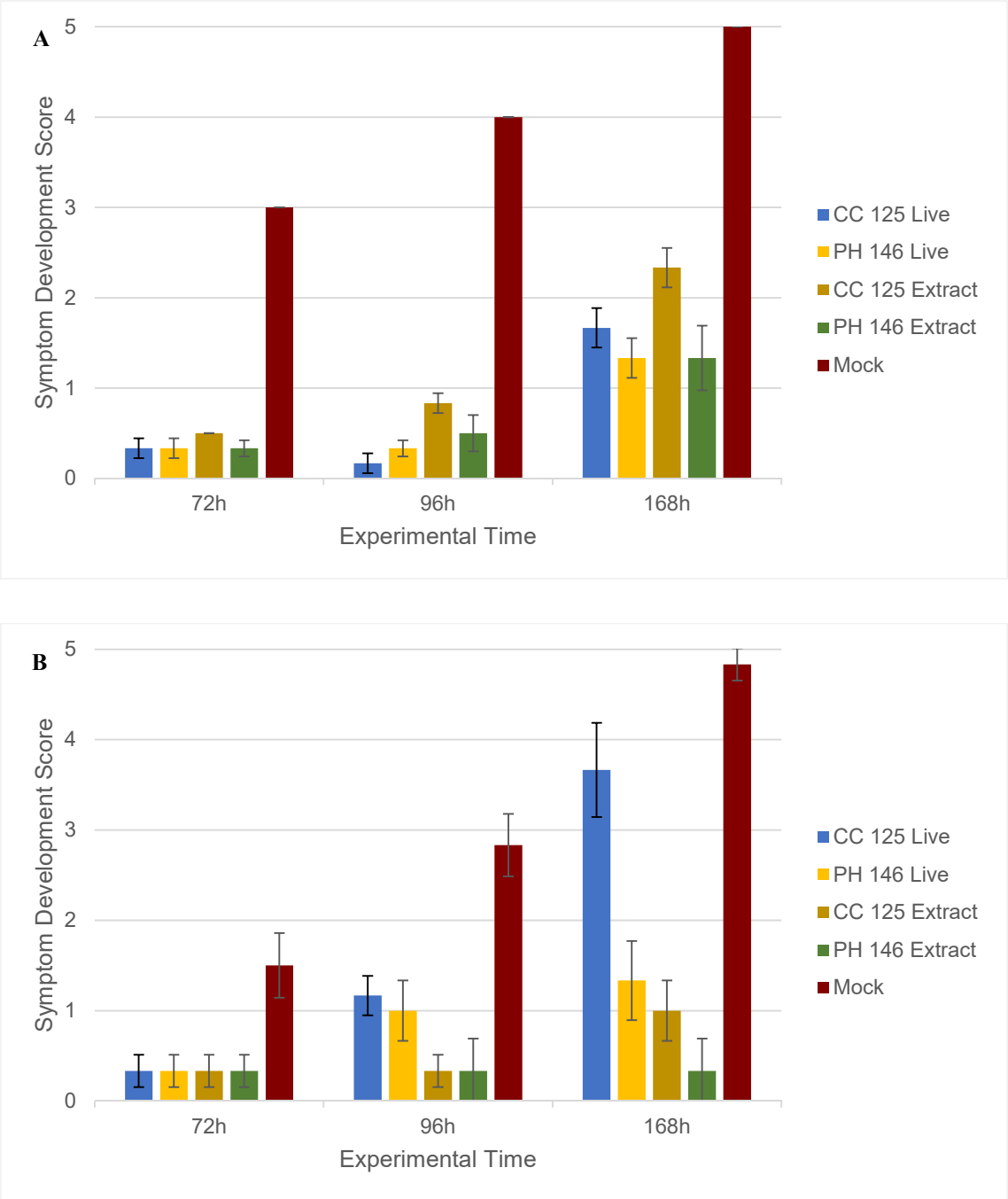


Figure 4.5: The symptoms observed in the NT leaves at 72h,96h and 168h of experimental time by scoring the patch of the leaf which was primed with the respective Chl strains CC 125 and PH 146 first, and then infiltrated with PstT. A. Experiment 1 and B. Experiment 2. The treated portions treated with CC 125 and PH 146 suspensions were not significantly different from each other (Unpaired Student's t-test, $p > 0.05$, n=5).

The symptoms induced by PstT in infiltrated leaves with CC 125 versus PH 146 were not that easily distinguishable from 72h to 168h of experimental time. The patches treated with CC 125 suspension and CC 125 extract were greener than the ones with PH 146 suspension and extract only at 168h

(while the patches looked equally green at 72 and 96h), while the mock treated patches had undergone complete necrosis by 168h (Figure 4.6).

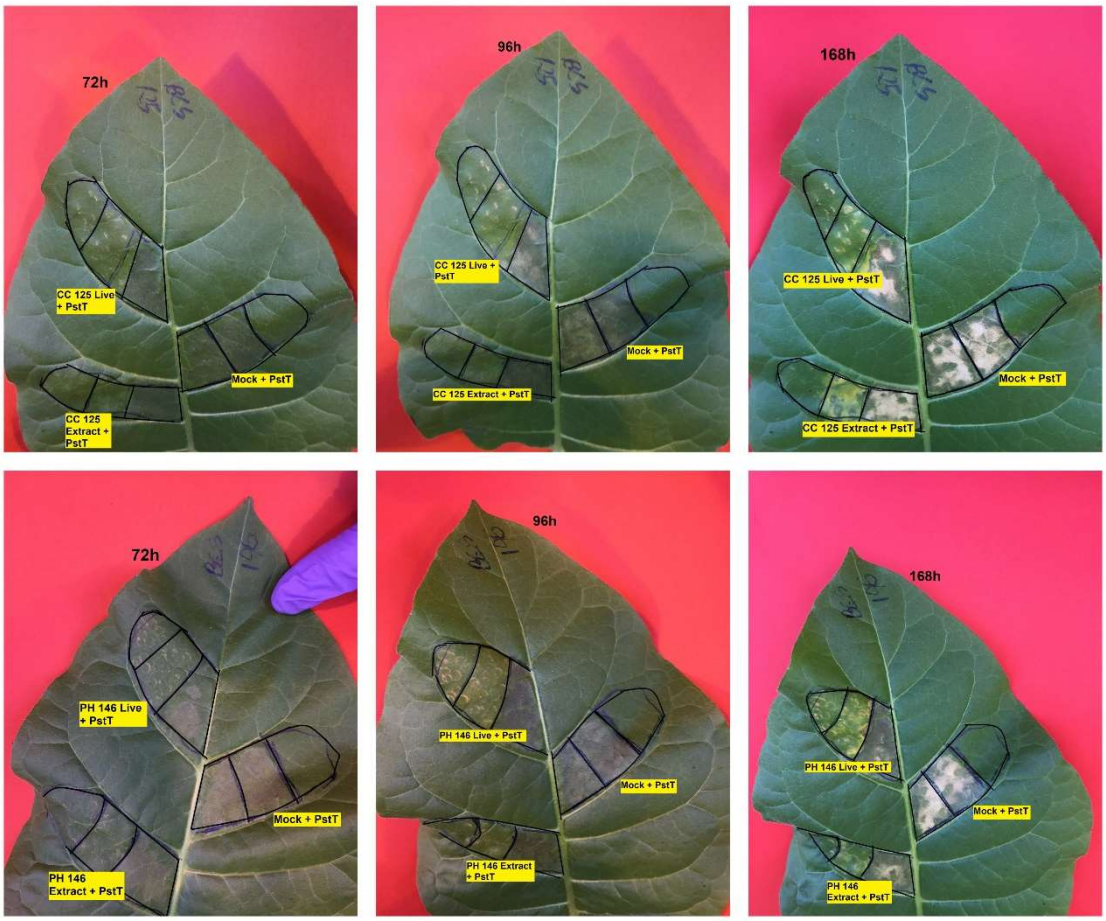


Figure 4.6: Examples of symptoms caused by PstT observed in NT leaves treated with WT CC 125 (above) and mutant PH 146 (below) at 72h, 96h and 168h experimental time.

In the first experiment done with CC 125 versus PH 147, the symptom development of PH 147 was compared against CC 125 and mock. In this assay we observed that both the suspension and the extract from PH 147 and CC 125 treated portions were not significantly different from each other and that the PH 147 mutant offered similar additional protection as the WT to the plant when compared with the mock treated patch of leaves (Figure 4.7A).

In the second experiment the symptoms in leaves treated with PH 147 (suspension and extract) were significantly more severe when compared to leaves treated with CC 125 (suspension and extract). Nevertheless, PH 147 mutant still gave some additional protection to the plant when compared with the mock treated patch of leaves (Figure 4.7B).

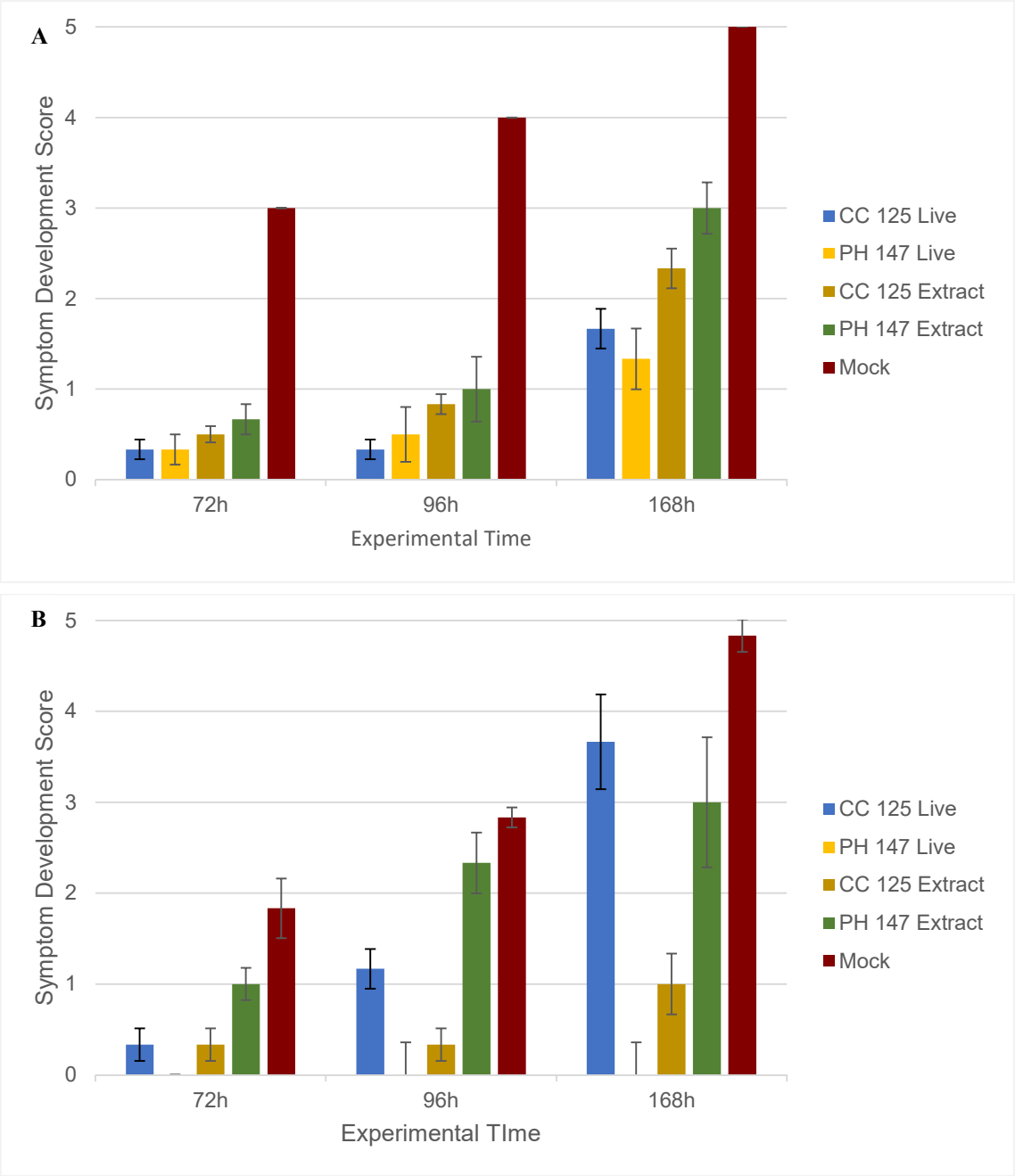


Figure 4.7: The symptoms observed in the *N. tabacum* leaves at 72h,96h and 168h of experimental time by scoring the patch of the leaf which was primed with the respective Chl strains CC 125 and PH 147 first, and then infiltrated with PstT at 48h experimental time. A. Experiment 1; B. Experiment 2. The mock, CC 125 live and PH 147 live treated portions are significantly different from each other, with the CC 125 treated portions showing the least severe symptoms (Unpaired Student's t-test, $p << 0.05$, n=5).

The symptoms induced by PstT in infiltrated leaves with CC 125 versus PH 147 are easily distinguishable from 72h to 168h of experimental time. The patches with CC 125 live and CC 125 extract are greener than the ones with PH 147 live and extract, while the mock treated patches had undergone complete necrosis by 168h (Figure 4.8).

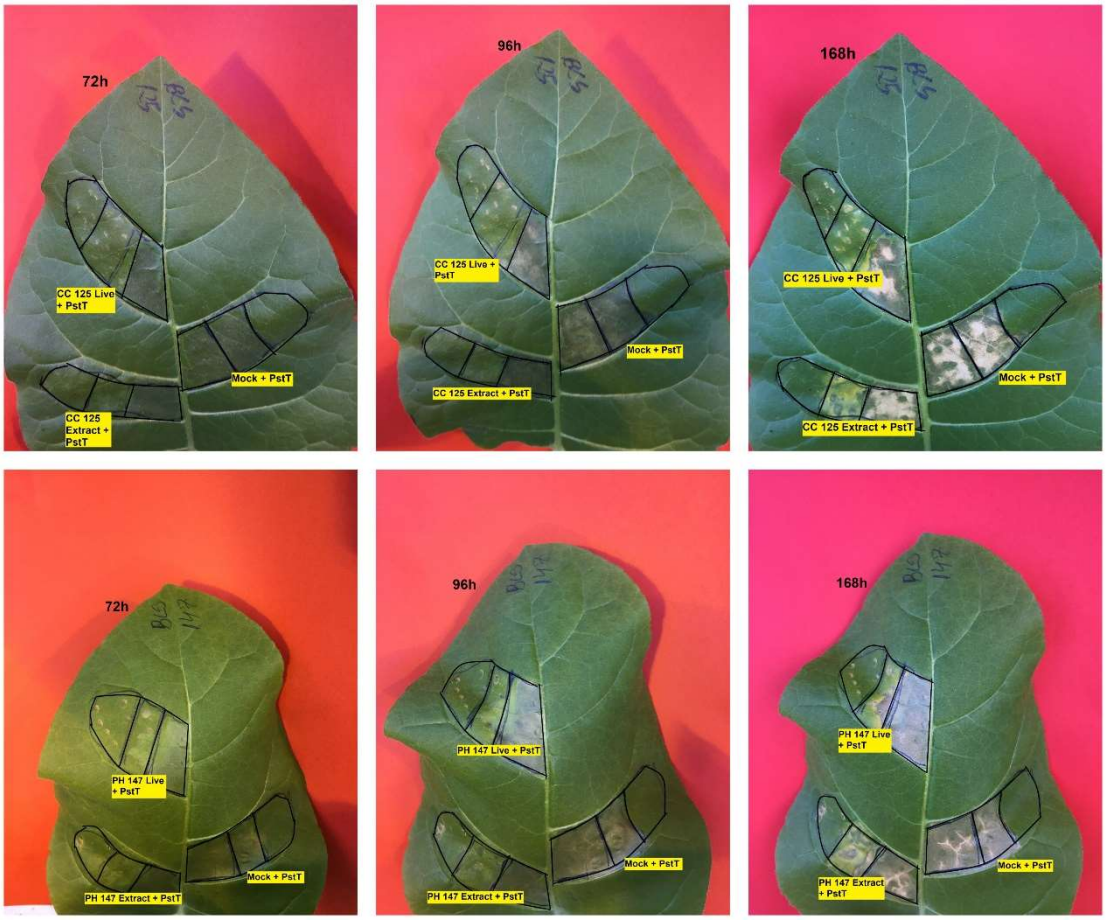


Figure 4.8: Examples of symptoms caused by PstT observed in NT leaves treated with WT CC 125 (above) and mutant PH 147 (below) at 72h, 96h and 168h experimental time.

4.1.2 WT CC-5325 versus cLOG KO

The Chl mutant 200648 is a knockout of a portion of the coding region of the Lonely Guy gene (LOG). There was only one infiltration experiment performed with WT CC 5325 versus 200648 mutant strain against PstT to track the symptom development. It was observed that the mutant 200648 showed significantly less symptoms than its corresponding WT CC 5325 (Figure 4.9).

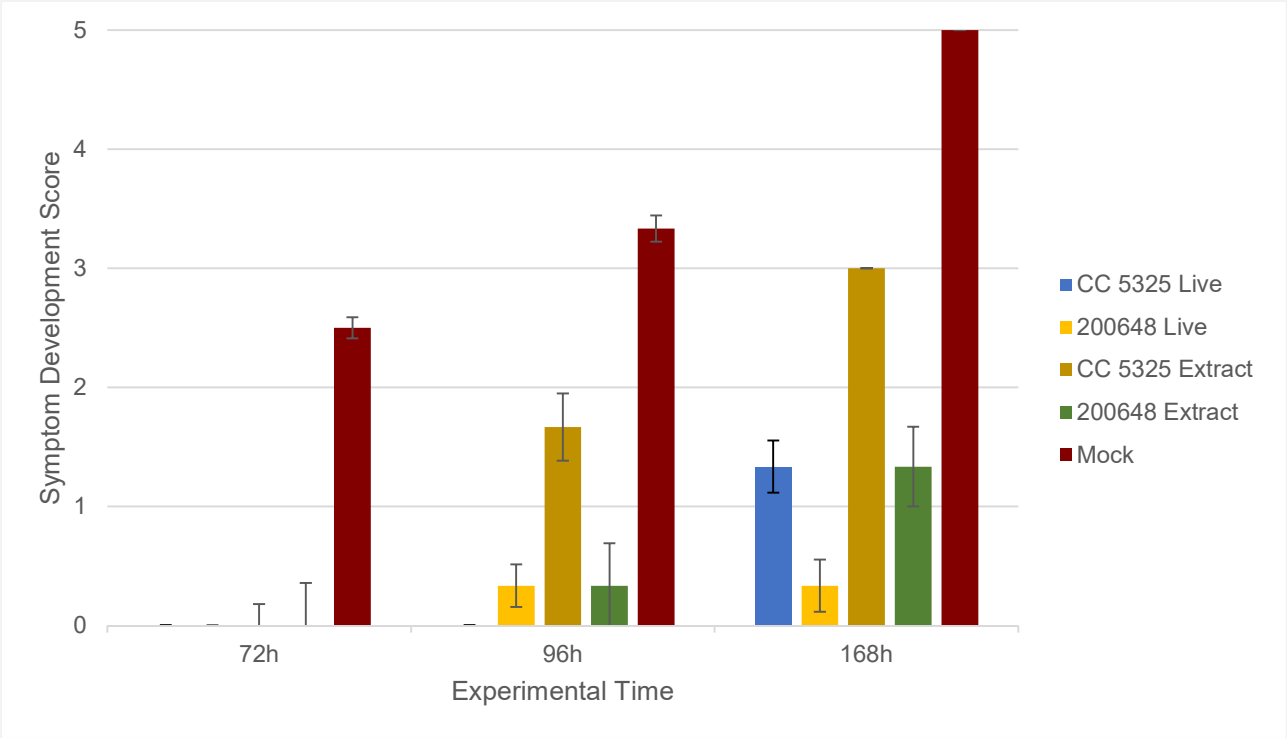


Figure 4.9: The symptoms observed in the NT leaves at 72h,96h and 168h of experimental time by scoring the patch of the leaf which was primed with the respective Chl strain first, and then infiltrated with PstT. The treated portions with CC 5325 and 200648 suspensions were significantly different from each other, with later showing less symptoms (Unpaired Student's t-test, $p < 0.05$, n=5).

The symptoms induced by PstT in infiltrated leaves with CC 5325 versus 200648 were easily distinguishable from 72h to 168h of experimental time. The patches infiltrated with CC 5325 and 200648 evidenced similar protection against the pathogen, while the mock treated patches had undergone complete necrosis by 168h (Figure 4.10).

Figure 4.10: Examples of symptoms caused by PstT observed in NT leaves treated with WT CC 5325 (above) and mutant 200648 (below) at 72h, 96h and 168h experimental time.

4.1.3 WT CC-5325 versus HMBPP KO

The Chl mutant 045820 is a knockout of a portion of the coding region of the HMBPP reductase gene (HMBPP). There was only one infiltration experiment performed with CC 5325 versus 045820 against PstT to track the symptom development. It was observed that the mutant 045820 showed significantly more severe symptoms than its corresponding WT CC 5325 (Figure 4.11).

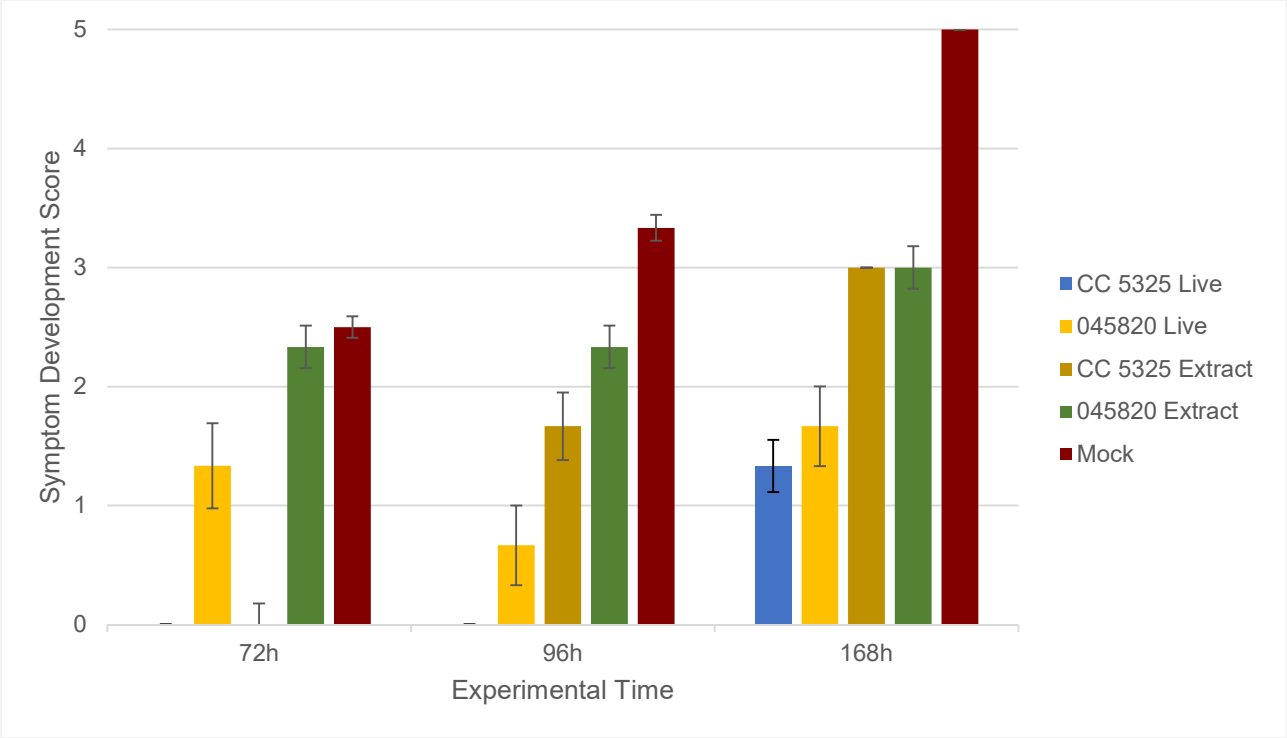


Figure 4.11: The symptoms observed in the NT leaves at 72h,96h and 168h of experimental time by scoring the patch of the leaf which was primed with the respective Chl strain first, and then infiltrated with PstT. The treated portions with CC 5325 and 045820 suspensions were significantly different from each other, with the first showing less symptoms (Unpaired Student's t-test, $p < 0.05$, n=5).

The symptoms induced by PstT in infiltrated leaves with CC 5325 versus 045820 were easily distinguishable from 72h to 168h of experimental time. The patches with CC 5325 suspension and CC 5325 extract were greener than the ones with 045820 suspension and extract, while the mock treated patches had undergone complete necrosis by 168h (Figure 4.12).

Figure 4.12: Examples of symptoms caused by PstT observed in NT leaves treated with WT CC 5325 (above) and mutant 045820 (below) at 72h, 96h and 168h experimental time.

In the trials where we tested the WT versus the mutants, the WT conferred a significantly higher protection than its corresponding 2-LOG KO mutants. A similar behaviour was observed between the CC 5325 WT and the HMBPP reductase KO mutant. On the other hand, a similar behaviour against PstT was observed between the WT and the cLOG KO mutant (Table 4.1).

Table 4.1: Summary of the effect of the various Chl treatments on symptom development of NT leaves infiltrated with PstT. Asterisks indicate probability levels (unpaired Student's t-test): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

	Total no. of trials with this strain	**No. of trials with <u>Stronger protection</u> (compared to control)**	**No. of trials with <u>Weaker protection</u> (compared to control)**	**No. of trials with <u>No protection</u> (compared to control)**
CC 125	6	6**	0	0
PH 144 (Crispr/Cas9 KO mutant)	2	0	2***	0
PH 145 (Crispr/Cas9 KO mutant)	2	0	2*	0
PH 146 (Crispr/Cas9 KO mutant)	2	0	2	0
PH 147 (Crispr/Cas9 KO mutant)	2	0	2*	0
CC 5325	2	1***	1**	0
045820 / HMBPP (CLiP KO mutant)	1	0	1***	0
200648 / cLOG (CLiP KO mutant)	1	1**	0	0

4.2 Priming with microalgae inhibited the proliferation of PstT in NT

Pst proliferation experiments were done in order to compare the proliferation of pathogen in leaf samples treated with microalgae (WT, mutants and mocks). The strains tested were the same as those used in the previous section.

4.2.1 WT CC-125 versus 2-LOG KOs

In the first set of experiments, the PstT concentration obtained in leaves treated with both PH 144 suspension and extract solutions were significantly higher when compared with the numbers obtained for CC 125 suspension and extract. The highest CFU/mL of PstT were always obtained from mock treatment (Fig- 4.13A). A similar trend was observed in the duplicate of this experiment (Fig. 4.13B).

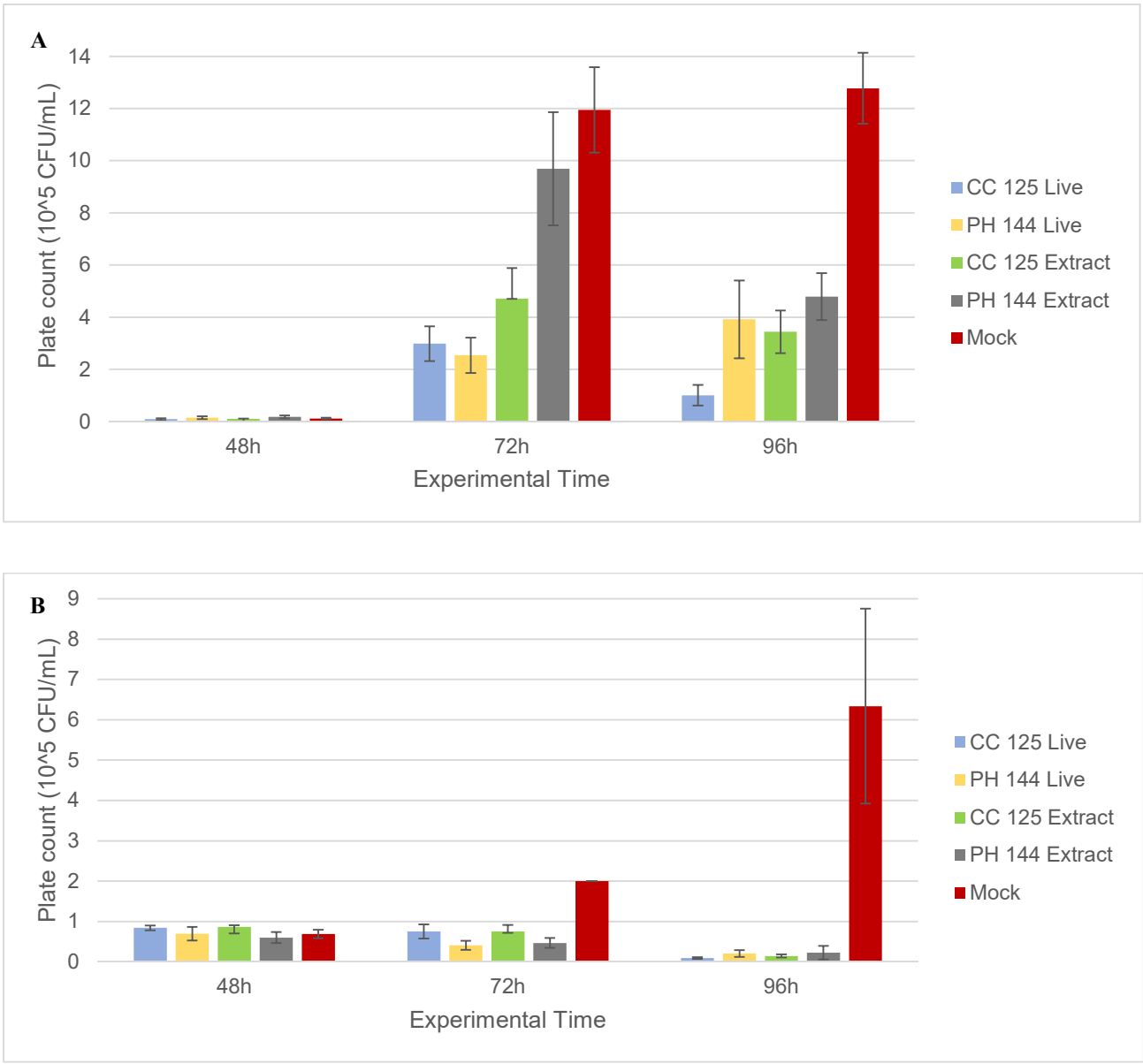


Figure 4.13: PstT numbers in NT leaf disks treated with CC 125 suspension, CC 125 Extract, PH 144 suspension, PH 144 Extract and Mock at 48h, 72h and 96h experimental time. A. experiment 1; B. experiment 2. The bacterial count in all these different treatments are significantly different from each other (Unpaired Student's t-test, $p << 0.05$, n=15).

In the first set of experiments, the PstT concentration obtained in leaves treated with both PH 145 suspension and extract solutions were significantly higher when compared with the numbers obtained for CC 125 suspension and extract. The highest CFU/mL of PstT were always obtained from mock treatment (Figure 4.14A). A similar trend was observed in the duplicate of this experiment (4.14B).

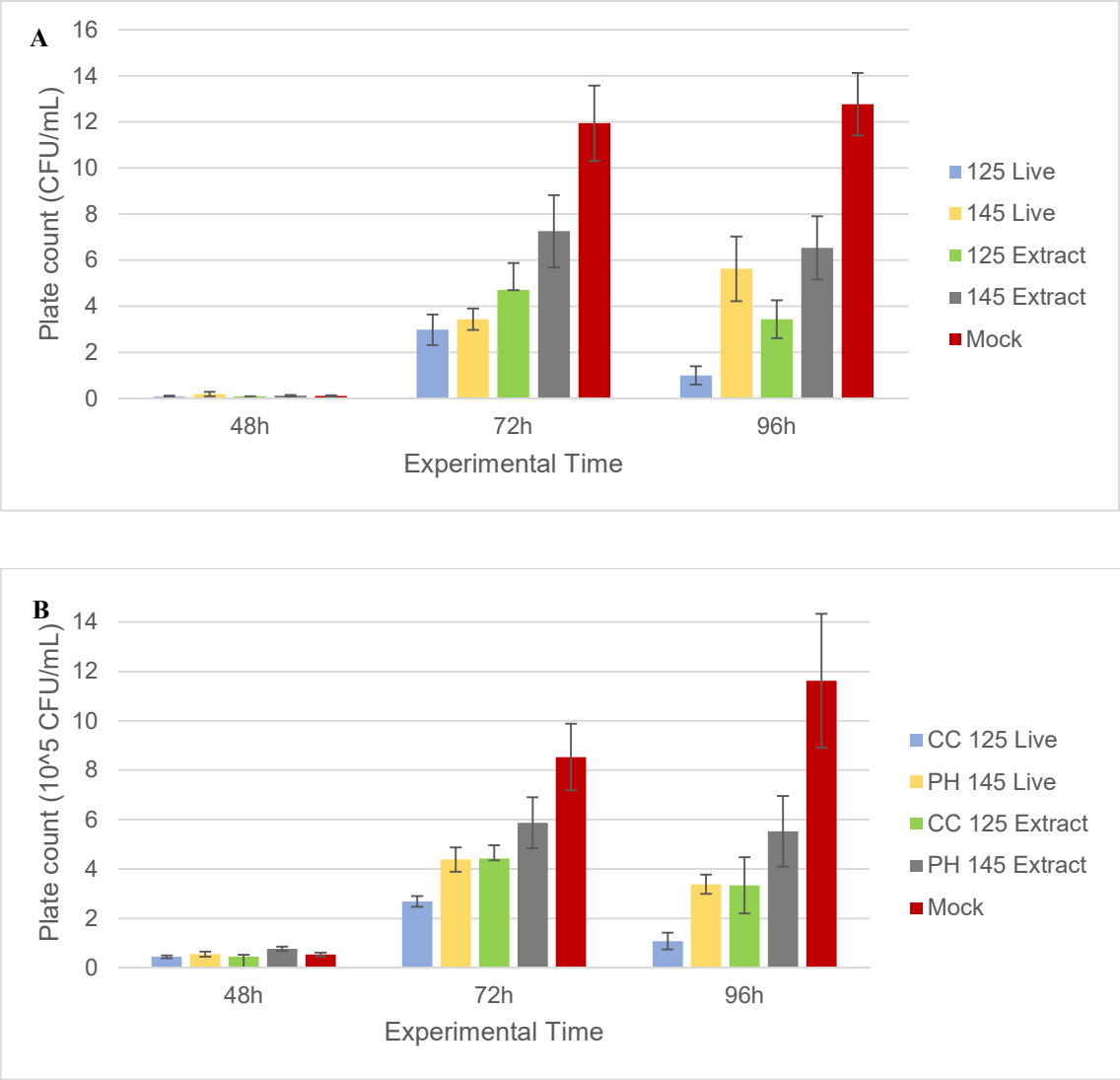


Figure 4.14B: PstT numbers in NT leaf disks treated with CC 125 suspension, CC 125 Extract, PH 145 suspension, PH 145 Extract and Mock at 48h, 72h and 96h experimental time. A. experiment 1; B. experiment 2. The bacterial count in all these different treatments are significantly different from each other (Unpaired Student's t-test, $p << 0.05$, n=15).

In the first experiment to compare the pathogen proliferation of CC 125 against PH 146, it was observed that the PstT count in both the PH 146 live solutions were significantly higher than the CC 125 live ones, but there was no significant difference between the CC 125 extract and PH 146 extract counts, while the mock always had the highest CFU/mL of PstT (Figure 4.15A). In the second repetition of this experiment, the mock, CC 125 live, extract and PH 146 live, extract was significantly different from one another (Figure 4.15B).

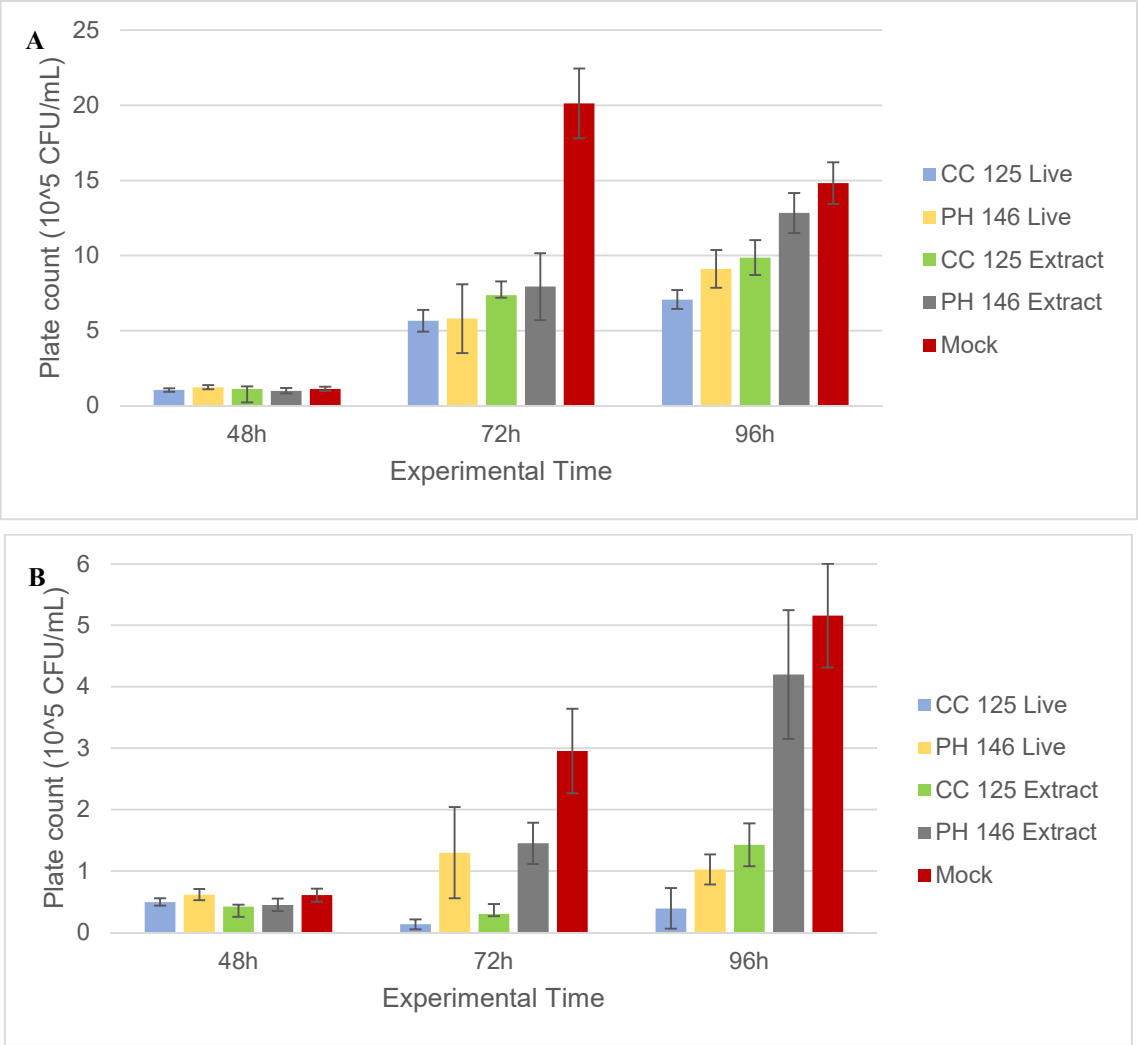


Figure 4.15B: PstT numbers in NT leaf disks treated with CC 125 suspension, CC 125 Extract, PH 146 suspension, PH 146 Extract and Mock at 48h, 72h and 96h experimental time. A. experiment 1; B. experiment 2. The bacterial count in all these different treatments are significantly different from each other (Unpaired Student's t-test, $p << 0.05$, $n=15$).

In the first experiment to compare the pathogen proliferation of CC 125 against PH 147, it was observed that the PstT count in both the PH 147 live and extract solutions were significantly higher than the CC 125 live and extract ones, while the mock always had the highest CFU/mL of PstT (Figure 4.16A). In the second repetition of this experiment, there was no significant difference between the PstT count in CC 125 live and PH 147 live, but all the other treatments had significantly different bacterial counts (Figure 4.16B).

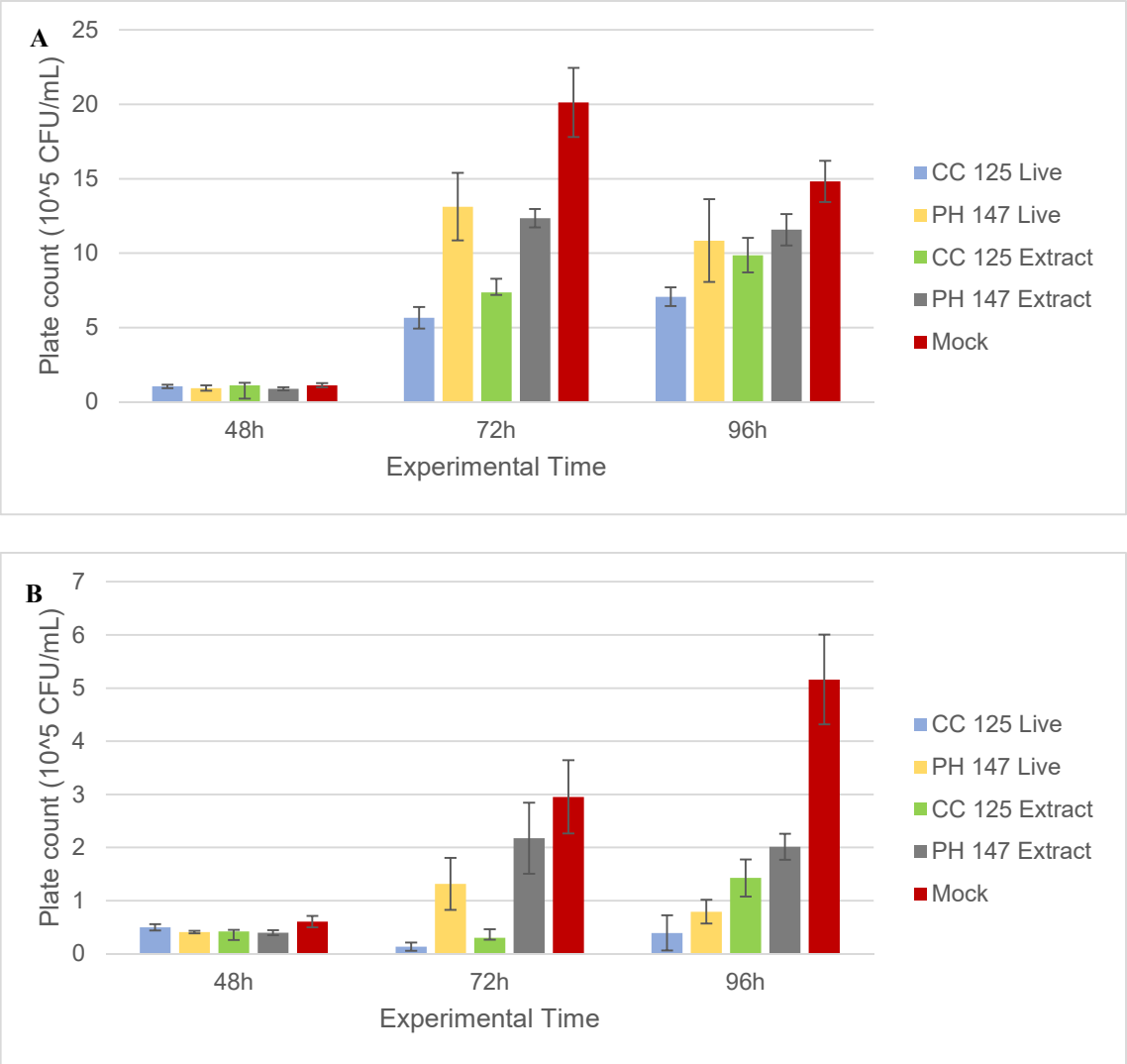


Figure 4.16B: PstT numbers in NT leaf disks treated with CC 125 suspension, CC 125 Extract, PH 145 suspension, PH 145 Extract and Mock at 48h, 72h and 96h experimental time. A. experiment 1; B. experiment 2. The bacterial count in all these different treatments are significantly different from each other (Unpaired Student's t-test, $p << 0.05$, n=15), except for CC 125 suspension versus the PH 147 suspension solution (Unpaired Student's t-test, $p << 0.05$, n=15).

4.2.2 WT CC-5325 versus cLOG KO

The PstT number obtained in leaves treated with 200648 suspension was significantly higher when compared with the numbers obtained for CC 125 suspension. On the other hand, no significant differences were obtained in Pst numbers between the extract's treatments. The highest CFU/mL of PstT was obtained from mock treatment (Figure 4.17).

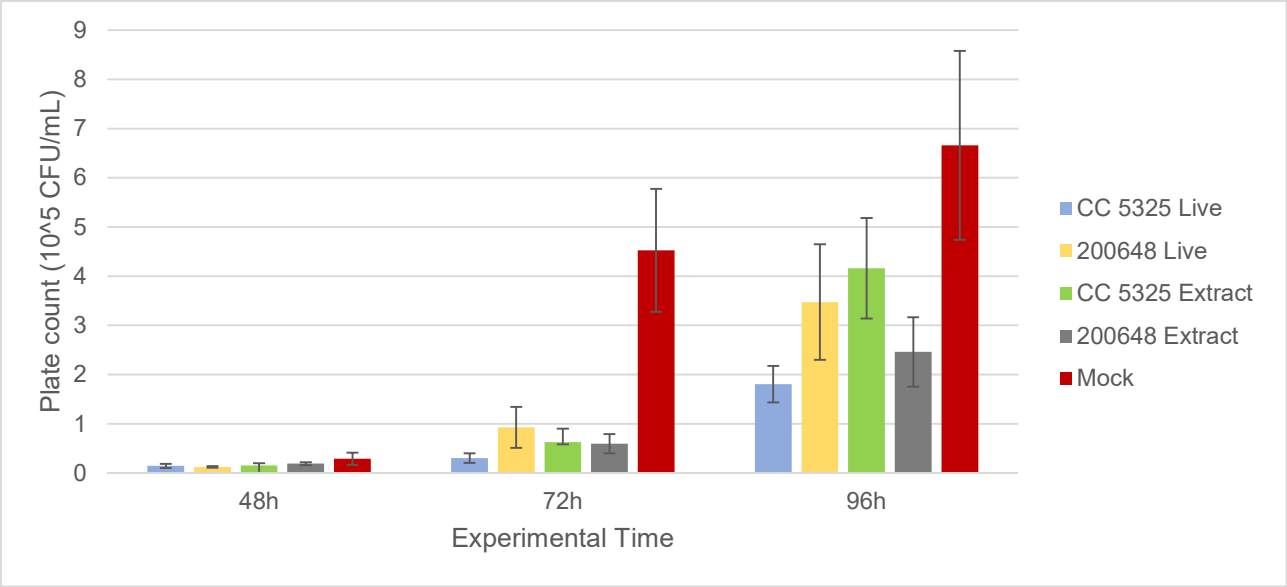


Figure 4.17: PstT numbers in NT leaf disks treated with CC 5325 suspension, CC 5325 Extract, 200648 suspension, 200648 Extract and Mock at 48h, 72h and 96h experimental time. The bacterial count in all these different treatments are significantly different from each other (Unpaired Student's t-test, $p << 0.05$, n=15)

4.2.3 WT CC-5325 versus HMBPP KO

The Chl mutant 045820 is a knockout of the HMBPP reductase gene (HMBPP). There was only one infiltration experiment performed with CC 5325 versus 045820 against PstT to track the pathogen proliferation of PstT in each of these treatments. In the experiment to compare the pathogen proliferation of CC 5325 against 045820, it was observed that the PstT count in both the 045820 suspension and extract solutions were significantly higher than the CC 125 suspension and extract ones, while the mock had the highest CFU/mL of PstT (Figure 4.18).

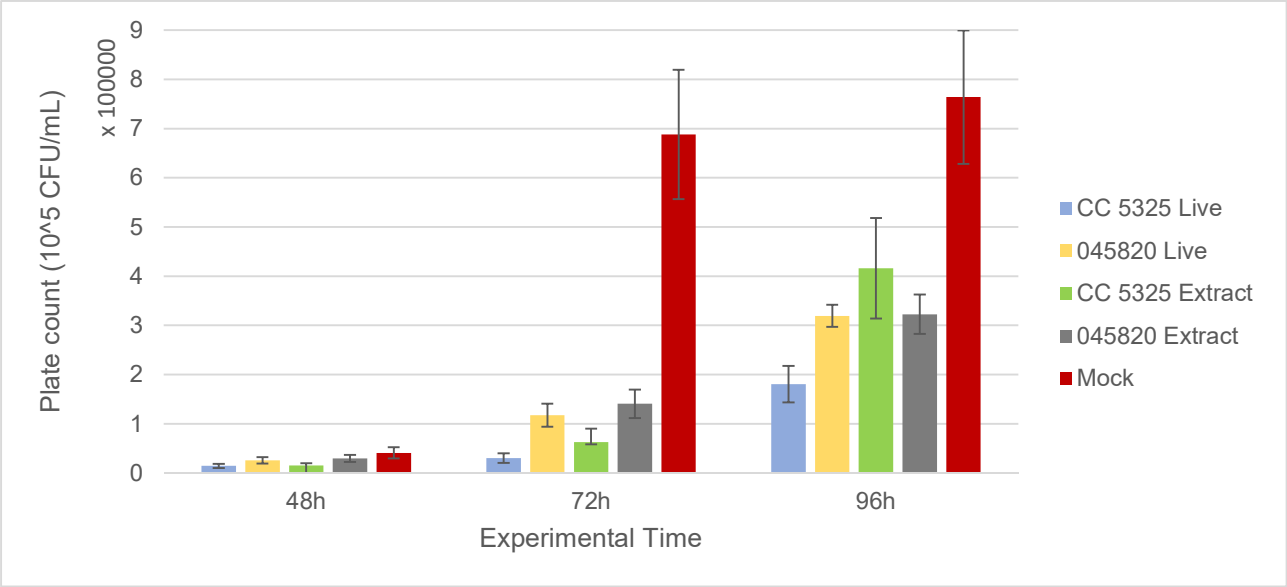


Figure 4.18: PstT numbers in NT leaf disks treated with CC 5325 suspension, CC 5325 Extract, 045820 suspension, 045820 Extract and Mock at 48h, 72h and 96h experimental time. The bacterial count in all these different treatments are significantly different from each other (Unpaired Student's t-test, $p << 0.05$, n=15)

In all the trials with all the wild types versus the knockouts, the wild types CC 125 almost always had a significantly lower PstT count than its corresponding 2-LOG KO mutants, and the wild type CC 5325 had a significantly lower PstT count than its corresponding KO mutants (Table 4.2).

Table 4.2: Summary of the effect of the various Chl treatments on bacterial count of PstT in *N. tabacum* leaves infiltrated with the said pathogen. Asterisks indicate probability levels (unpaired Student's t-test): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. No asterisks indicate no significant effects.

	Total no. of trials with this strain	**No. of trials with <u>Stronger protection</u> (compared to control)**	**No. of trials with <u>Weaker protection</u> (compared to control)**	**No. of trials with <u>No protection</u> (compared to control)**
CC 125	6	6**	0	0
PH 144 (CRISPR/Cas9 KO mutant)	2	0	2***	0
PH 145 (CRISPR/Cas9 KO mutant)	2	0	2***	0
PH 146 (CRISPR/Cas9 KO mutant)	2	0	2**	0
PH 147 (CRISPR/Cas9 KO mutant)	2	0	2**	0
CC 5325	2	2**	0	0
045820 / HMBPP (CLiP KO mutant)	1	0	1***	0
200648 / cLOG (CLiP KO mutant)	1	0	1*	0

4.3 Microalgae extract showed no *in vitro* antimicrobial activity against PstT

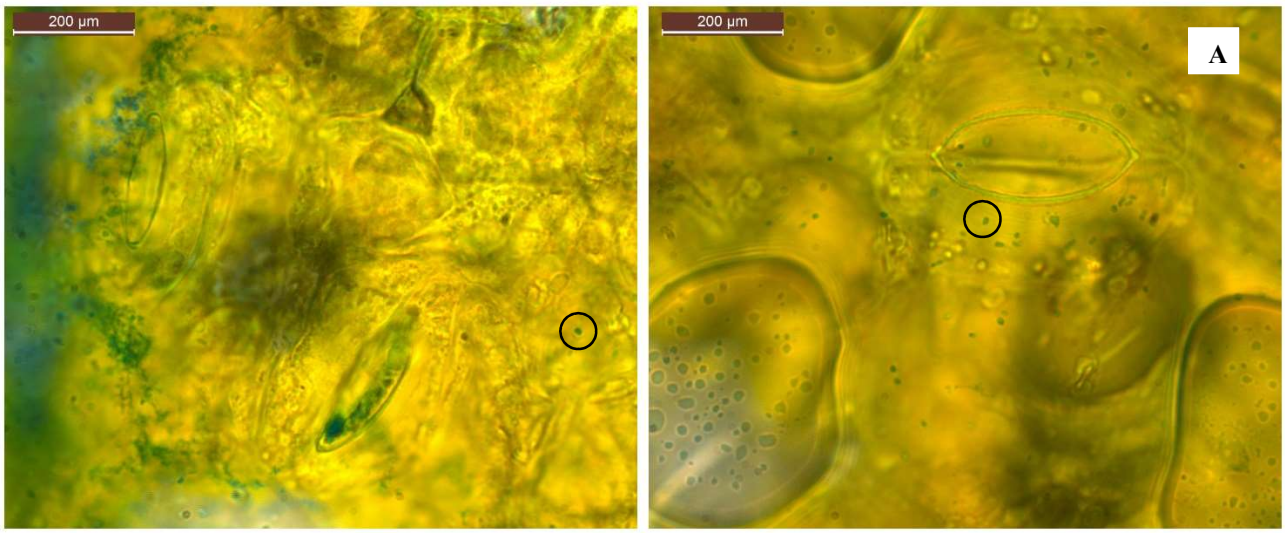
The screening for antimicrobial activity was performed in order to determine if the microalgae treated leaf portions could inhibit the growth of PstT *in vitro* as they did in the plant. To test the antimicrobial activity of the various leaf extracts treated with microalgae against PstT, various extraction procedures were carried out followed by broth dilution and disk diffusion assays. However, there were no growth inhibition of PstT by these extracts, neither of the kinetin treated positive controls (Appendix 1).

4.4 Microalgae inhibited pathogen multiplication in NT

The leaf tissues primed with the WT strain CC 5325 and then infected with PstT were compared to the leaf tissues primed with water (mock) and then infected with PstT. The comparison was performed under the microscope in order to see if the bacteria survived in the plant when treated with microalgae, even if they did not show any symptoms when observed with the naked eye.

Microscopy was done by staining the NT leaf sections primed with CC 5325 strain and mock with aniline blue up to 4 days post infection (DPI). This allowed tracking PstT in the leaf since it appeared as blue dots under the Leica DM 2000 microscope at 100x magnification.

At one DPI several PstT cells were observed in leaves primed with CC 5325 strain and mock (Figure 4.19A). At two DPI, lesser PstT cells were observed in leaves primed with CC 5325, while there seemed to be almost the same number of PstT in the mock samples (Figure 4.19B). Ultimately, by four DPI, there were almost no PstT cells in the CC 5325 samples, and the ones observed were elongated and tubular; while in the mock there were more PstT cells observed which were clumped together (Figure 4.19C).



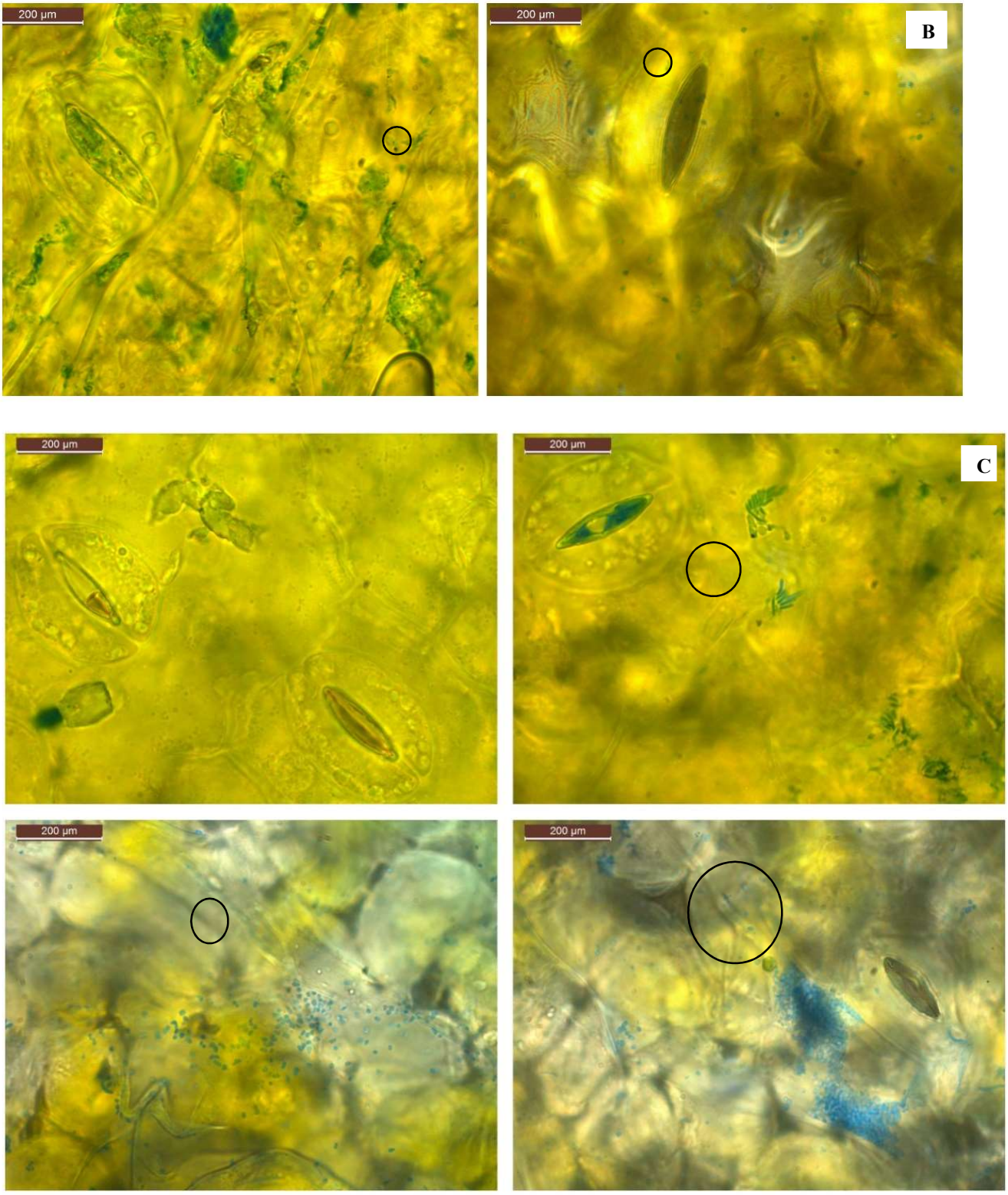


Figure 4.19: -NT leaves primed with CC 5325 and then infiltrated with PstT (left) vs primed with water (mock) and then infiltrated with PstT (right) seen under a Leica DM 2000 Microscope at A.one DPI B. two DPI C. three DPI, 100x magnification. Staining of the bacteria has been done with aniline blue. The PstT appear as blue dots or elongated structures (as inside black circles) when stained with aniline blue.

4.5 Microalgae induced drought tolerance in *Arabidopsis*

The ability of the WT CC 125 strain to confer resistance to drought was tested in *Arabidopsis* plants as described in section 3.13.

After 7 days of drought (the plants were not watered), the weight of the well-watered plants supplemented with CC 125 suspension, CC 125 extract and CC 125 culture was significantly higher when compared to the control (Figure 37). The weights of the drought treated plants were highly variable among them and the differences were not significant (Figure 4.20).

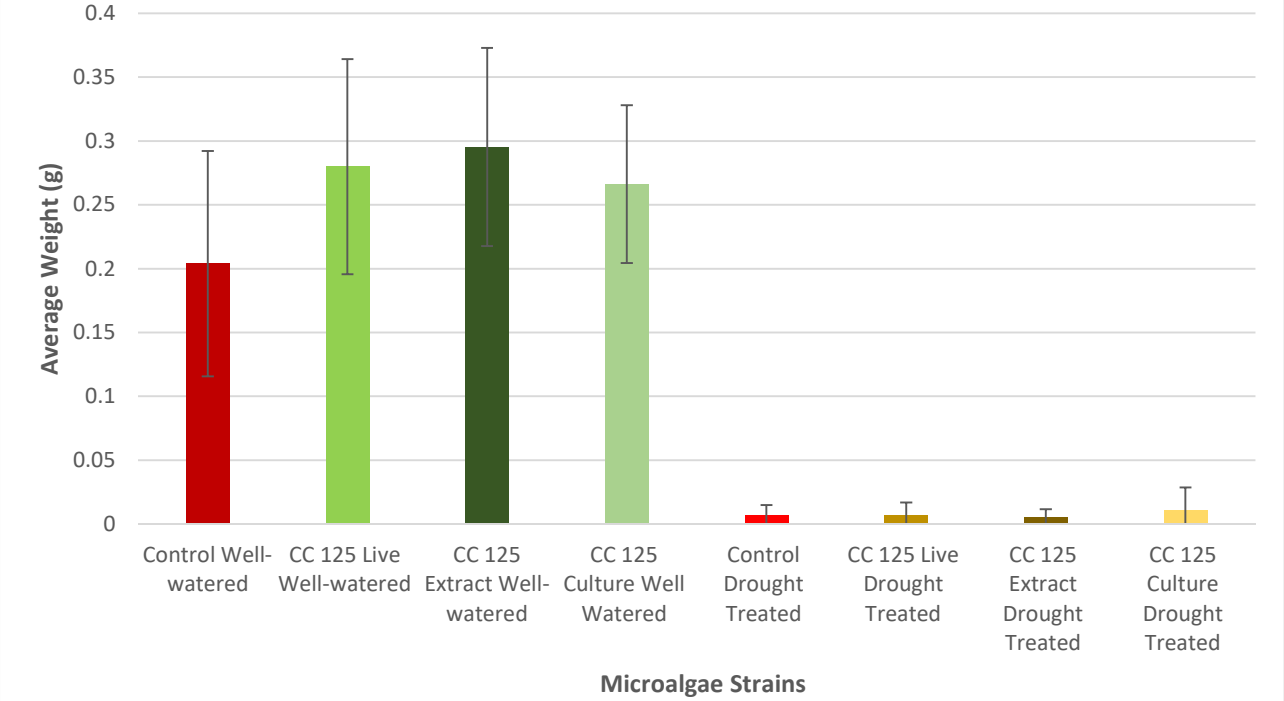
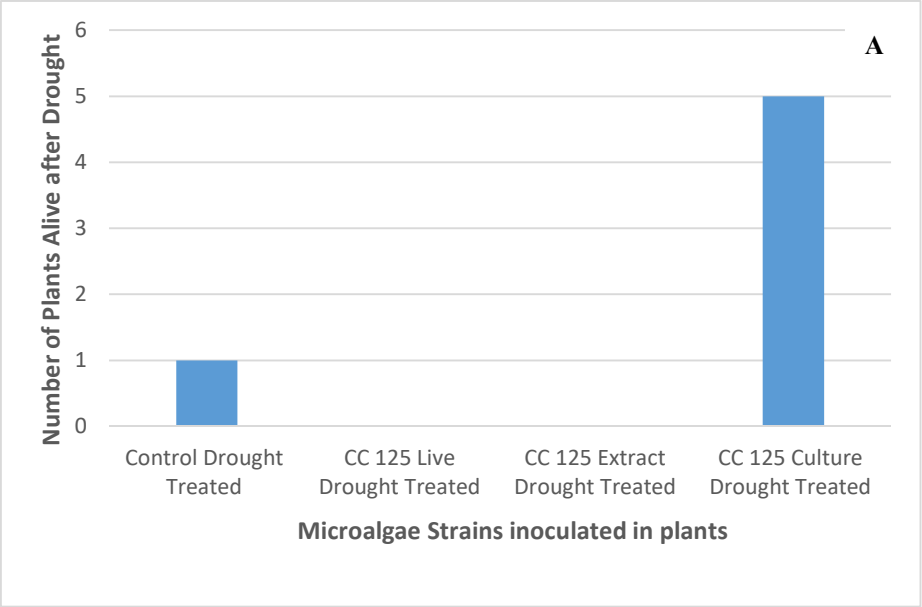


Figure 4.20: Drought tolerance experiment results on *Arabidopsis* plants primed with different solutions of CC 125 microalgae. Well-watered plants supplemented with CC125 had a significantly higher growth than the control ones (Unpaired student's t-test, $p << 0.05$, $n=36$) . In the drought treated ones, only the CC 125 culture seems to have a higher growth than the control, but it is not significant, with a very high amount of standard deviation in the weight between plants (Unpaired student's t-test, $p > 0.05$, $n= 36$).

Among the plants subjected to the drought treatment, 5 out of the 36 plants treated with CC 125 culture survived after 7 days, while only one of the control plants survived, and none of the CC 125 live or CC 125 extract treated plants survived the drought assay (Figure 4.21A, B).



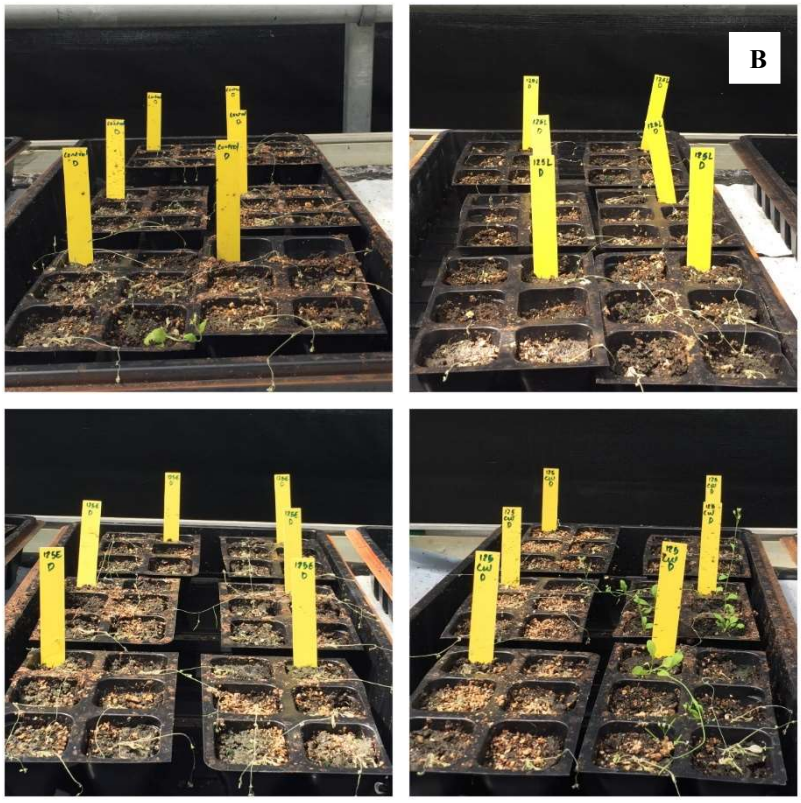


Figure 4.21: A. Number of plants which survived a drought of 7 days out of the given treatments. B: Five out of 36 plants treated with CC 125 microalgae culture (lower right) survived the 7-day drought, while one out of 36 plants survived in the control ones (upper left). However, none of the CC 125 live or CC 125 extract treated plants survived the drought (upper right and lower left respectively).

The average SWC of the soils between the different treatments was also measured. Only the well-watered soils treated with CC 125 suspension had a significantly higher SWC than the well-watered control, while the CC 125 extract and CC 125 culture treated, and drought treated soils had a significantly higher SWC than the drought treated control (Figure 4.22).

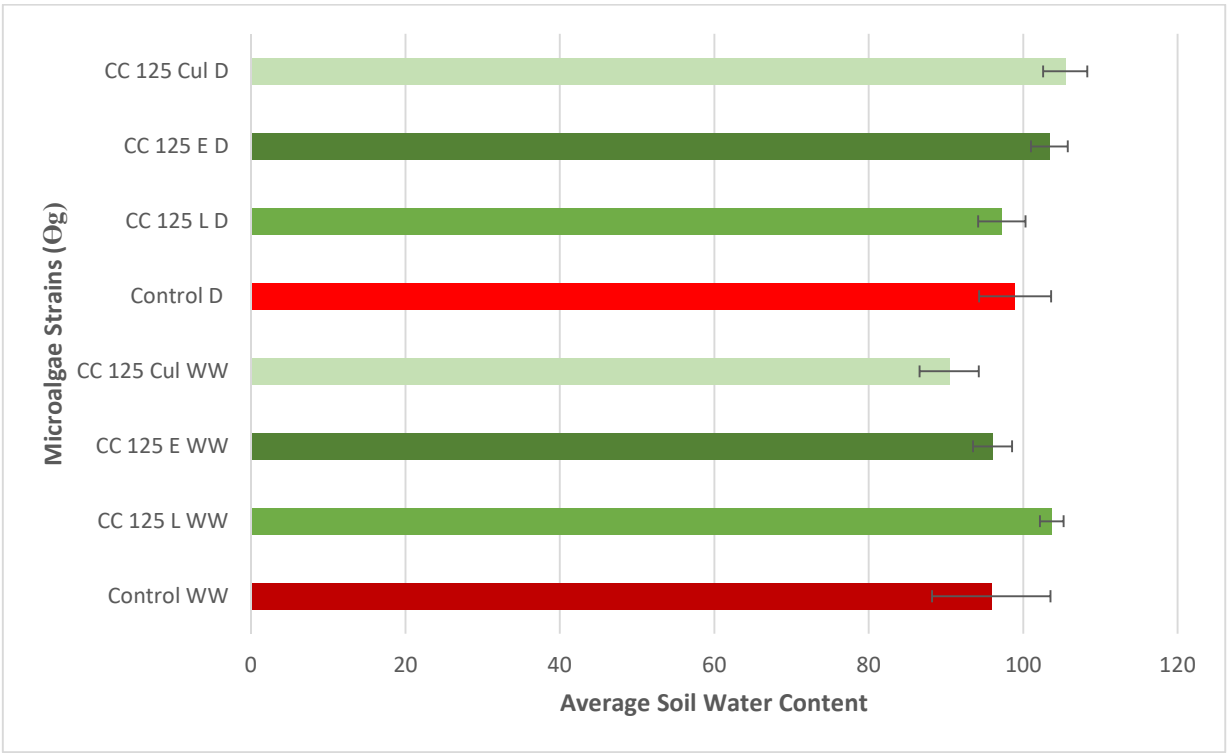


Figure 4.22: Average soil water content (SWC) of plants subject to various microalgae treatments under well-watered (WW) and drought treated (D) conditions. The CC 125 live treated well-watered soils had a significantly higher SWC than the well-watered control, while the CC 125 extract and CC 125 culture treated and drought treated soils had a significantly higher SWC than the drought treated control (Unpaired Student's t-test, $p < 0.05$, n=6).

4.6 Microalgae promoted tomato seed germination

The ability of microalgae strains CC 125, PH 144, PH 147, CC 5325, 200648 and 045820 to promote tomato seed (variety Moneymaker) germination was tested.

Four days after inoculating the seeds with the respective treatment, the germination percentage of PH 144 supernatant and PH 147 supernatant was the highest. All the other treatments presented a higher germination percentage than the control, except for the CC 125 suspension, CC 125 supernatant, CC 5325 suspension, 200648 live, 200648 extract with an apparently inhibitory effect and 045820 live with a negative impact in treated seeds (Figure 4.23).

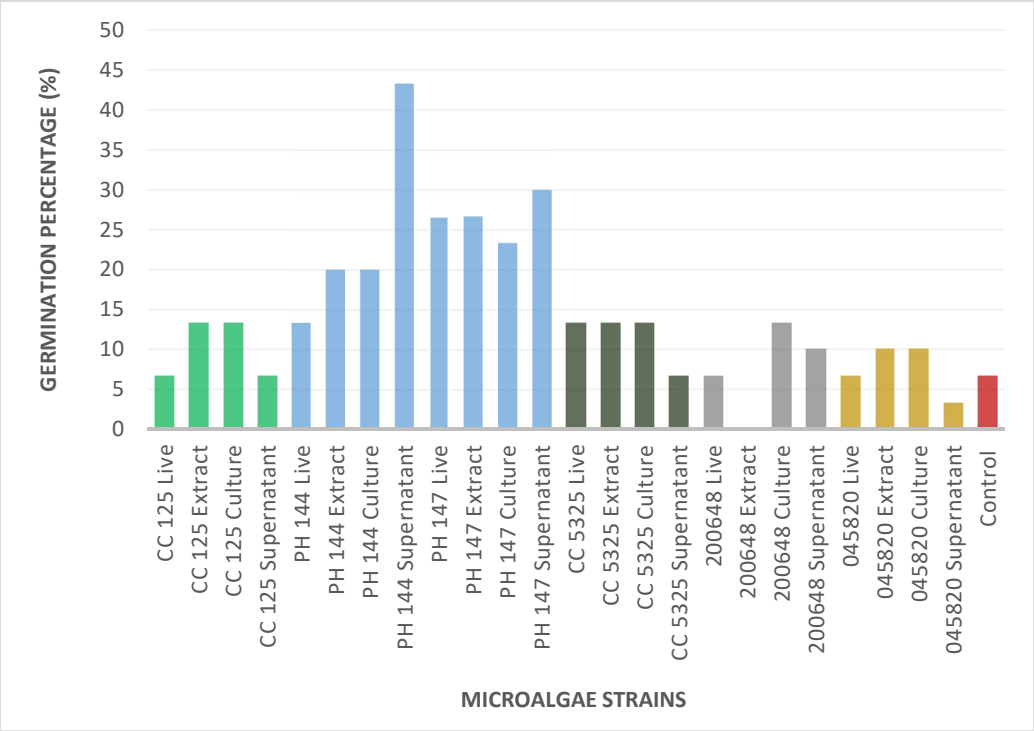


Figure 4.23: Percentage of germinated tomato seeds (var. moneymaker) from each treatment solution four days after inoculation with microalgae (n=30).

Five days after inoculating the seeds with microalgae the results were similar as the previous day for PH 144 supernatant and PH 147 supernatant treated seeds having the highest germination percentages; while CC 125 live, 200648 live, 200648 extract, 045820 live and 045820 supernatant had lower or equal germination percentages as the control (Figure 4.24). Another interesting observation was that not only did the microalgae treated seeds germinate faster than the control, but they also grew longer radicals (Figure 4.25). The negative impact of live 045820 treatment was most notorious (Appendix 2).

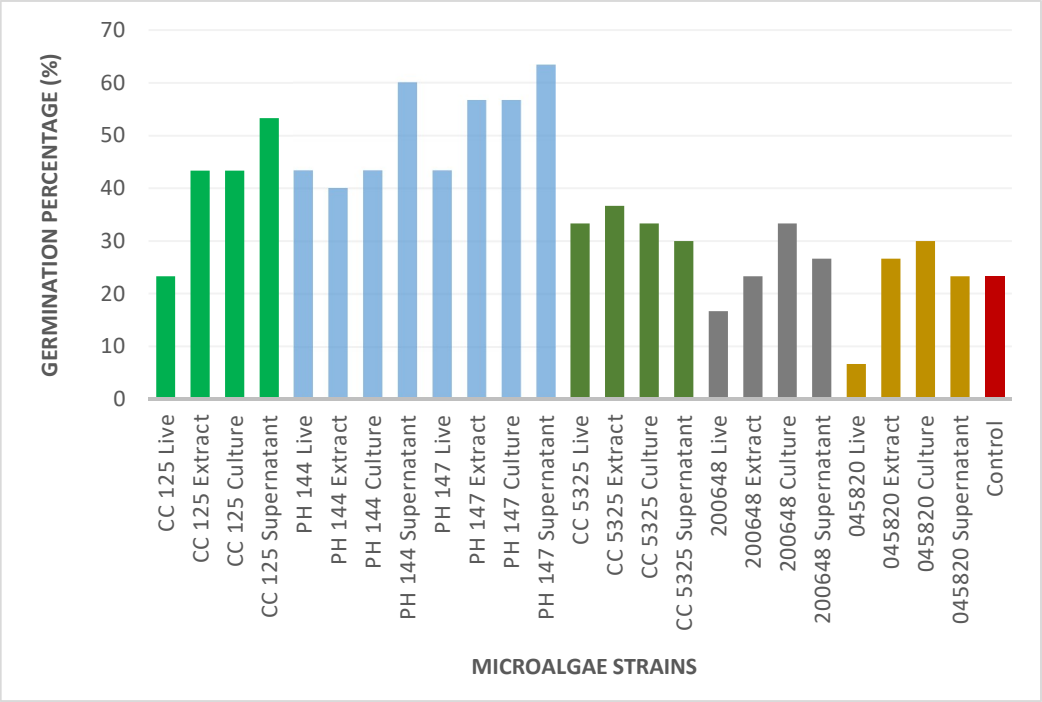


Figure 4.24: Percentage of germinated tomato seeds (var. moneymaker) from each respective treatment solution five days after inoculation with different microalgae strains (n=30).

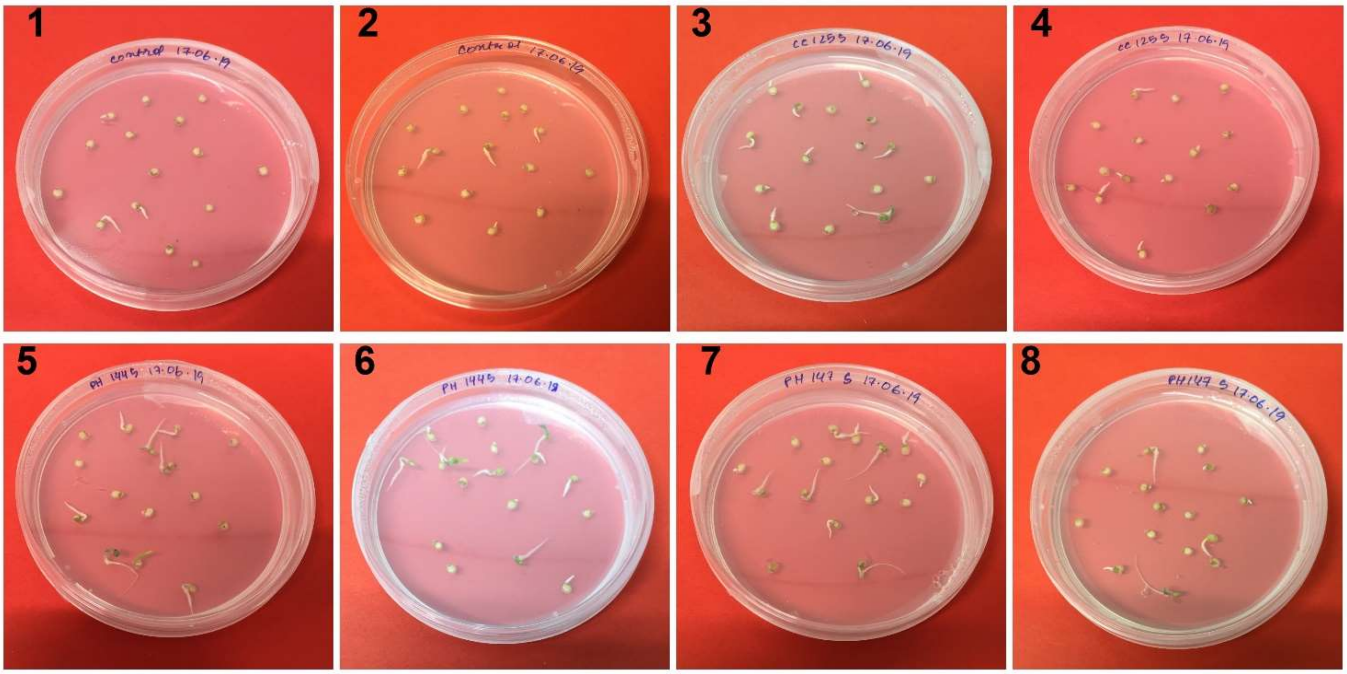


Figure 4.25: Germinated tomato seeds in Petri dishes five days after inoculation with water (1,2), CC 125 supernatant (3,4), PH 144 supernatant (5,6) and PH 147 supernatant (7,8).

4.7 Microalgae promoted the growth of NT and tomato

Growth assays were performed on NT and tomato (variety Gartenfreude) with CC 125 live, CC 125 extract and CC 125 culture solutions, with 10 biological replicates for each treatment. The CC 125 live treated plants were observed to have the significantly high weight amongst these treatments, while the CC 125 extract treated plants had significantly lower weights than the control (Figure 4.26).

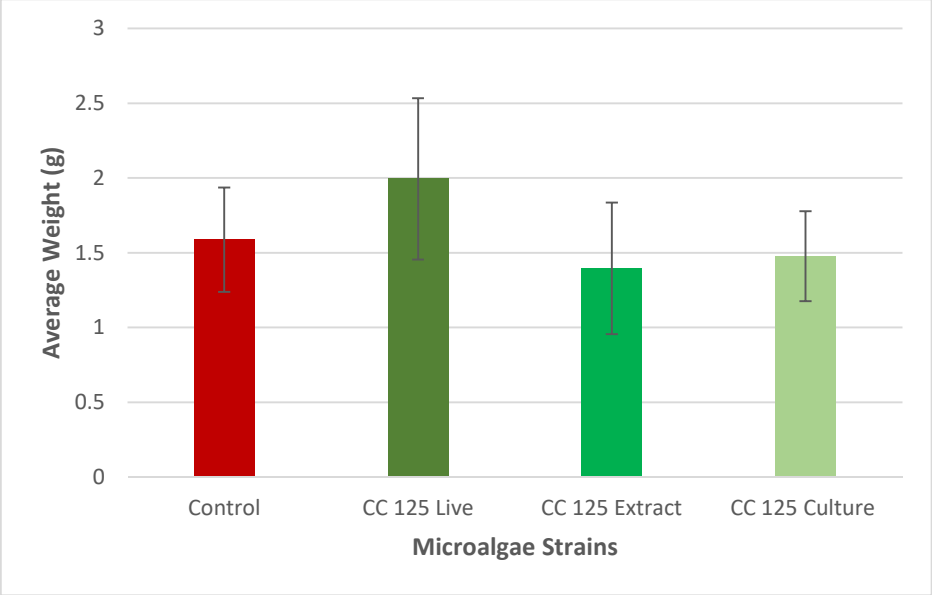


Figure 4.26: Comparison between the average weights of NT plants inoculated with different treatments of CC 125 solutions at their seedling stage. CC 125 live solution treated plants had a significantly higher weight than the control plants, while the CC 125 extract treated plants were significantly lower than the control ones (Unpaired Student's t-test, $p < 0.05$, n=10)

The CC 125 treated plants were not only heavier than the control ones, but they also had bigger shoots with more leaves and longer roots which were more branched (Figure 4.27). In the case of tomato, there were no significant results obtained with respect to the different microalgae treatments administered to tomato plants when compared to the control (Appendix 3).

Figure 4.27: Comparison between a tobacco seedling previously treated with water (left) and CC 125 live solution (right). Not only has the plant shoot treated with CC 125 live solution grown taller, but the roots are also longer and more branched

5. Discussion

The objectives of this study included the functional characterization of the interaction between organisms in which the production of CKs by microalgae putatively strengthens the plant defence system of *Nicotiana tabacum* (NT). It was also intended to determine if these microalgae could perform functions as a bio-stimulant in other crops. This study showed that the production of CKs by the *Chlamydomonas reinhardtii* (Chl) microalgae strengthens the plant's defence responses, as the wild type Chls gave greater protection to NT plants against *Pseudomonas syringae* pv. *tomate* (PstT) compared to the mutants in which the HMBPP reductase gene and the LOG gene were eliminated. Regarding the bio-stimulation or bio-fertilisation capacity of algae, wild type Chl strains were found to be successful in stimulating the growth of tomato, tobacco and *Arabidopsis* plants in their sowing and seedling phases. These preliminary results predict the potential protective function of these algae against abiotic stresses, such as drought in Arabidopsis production. In addition, both the Chl mutants and the wild types of this microalgae have been shown to have a positive impact on the germination of tomato seeds.

5.1 Algae solutions

Many studies have been performed on the effect of different algal compounds infiltrated in leaves of live plants or mixed in the soil (Renuka et al. 2018), but no studies have been performed on the effect of infiltrating the leaf of a plant with live algae and/or algal extracts that contained disrupted algal cells. Most of the research on this area focuses on the compounds themselves, and the specific algal compounds are infiltrated after purification (Mercier et al. 2001). Hence, the approach used in this work was new trying to shed some light on the effects that live algae would have on plants and on their defence responses.

The infiltration methodology was originally designed for bacteria, so carrying it out was complicated and time-consuming since algae are considerably bigger in size than bacteria. Due to the algae size, infiltration of the algae could affect the plant negatively and cause mechanical damage. It is hypothesized that at the early stages of infection *Pseudomonas syringae* forces the plant cells to leak nutrients, which help the pathogen to grow and ultimately kill the plant cells (Katagiri, Thilmony, He 2002). Infiltration with algae could in this case create better conditions for the pathogen, since these conditions mimic the early infection steps by PstT. Also, the biomass of the algae inserted into the

leaves was very large (75g/L), which could overload the infiltrated area as algae produce a large number of compounds (Michalak, Chojnacka 2015).

Algae extracts were also used to infiltrate the leaves, and they included everything that was present in the algae at the time in a slur of different phytohormones and lipids, etc. The algal extracts were produced by microwaving them, but other methods of cell disruption have also proved to be successful (Lee et al. 2010; McMillan et al. 2013). Despite all limitations that could arise with infiltrating the leaves of the plants with algae extracts, the algae solutions were successful in providing additional resistance to the NT plants against PstT. The solutions with live microalgae provided better protection to the plants than the extracts. Both live microalgae and extract solutions protect the plant against PstT attacks. Despite this, the protection provided by live microalgae has been superior, which may be related to the degradation of important compounds in the extract, or to the synthesis of compounds by live algae during the experiment.

5.2 Pathogen resistance promotion in NT

Infiltration experiments to test the PstT induced symptom development and pathogen proliferation in NT leaves treated with microalgae produced robust and reproducible results where all the microalgae strains strengthen the plants' defence responses against PstT.

The leaves of NT are large and sturdy, which made it easy to track the symptoms over a course of a few days after the infiltration with PstT. However, the only problem with the symptom development experiment was that since it was based on a scoring system, it could produce different results if different people performed it, hence experimenter's bias could occur with the symptom development experiments. Also, the symptom development was shown to vary vastly in potency from experiment to experiment. This may be due to the environmental changes which occurred from the beginning of the project (such as changes in temperature, humidity etc) compared to the end. In addition, in future experiments it could be an improvement to optimize for an ideal OD to infiltrate the plants, to achieve a higher and more sensitive definition of the symptom development from day to day.

However, the robust results of the symptom development experiments are complemented and supported by the pathogen proliferation experiments, where the mock leaf disks repeatedly had much higher counts of PstT than the microalgae treated leaf disks. Interestingly, in the pathogen proliferation experiment the mock treatment showed noticeably higher CFU from even 48h experimental time, which suggest that the antimicrobial properties of the microalgae cause immediate

effects. This may also be due to how the method was performed, as the samples are leaf discs inside which pathogens have infiltrated. These leaf discs are grinded and then the pathogen is plated and incubated for 24 hours. This results in the pathogen being exposed to the antimicrobial contents of the leaf for 24 hours, which may have skewed the results a bit.

Although many factors could contribute to this resistance promotion by algae, only the CK-induced impact was investigated through this experiment. The Chl-mutants with impaired CK signalling and production were not as effective as a pathogen resistance promotor as the WT. The differences between the CC 5325 and HMBPP-KO strongly hinted that the CK production in the algae was important for induced resistance. Furthermore, the 2-LOG mutants and cLOG mutants with impaired CK activation also proved to have reduced effects on the plant-pathogen interaction. This shows that for the algae to successfully promote pathogen resistance active forms of CK are needed. And while other phytohormones in the algae might also contribute to the resistance, without CK the resistance will not be induced as properly.

This goes hand in hand with previous work by Großkinsky and colleagues in which they described a method by which they could induce pathogen resistance in plants using CK producing microorganisms, and it was further proved that the CK production in this bacteria was the cause of the resistance towards the pathogen DC3000, after the creation of loss of function and regain of function mutants (Großkinsky et al. 2016).

Our results further broaden the scope in which CKs could be used in pathogen resistance. To understand these results further, a hormone analysis of the different mutants in comparison with the WT might shed light on these interactions.

5.3 Chl-mutant strains testing

Chlamydomonas reinhardtii was chosen for this study because despite being a high CK production microalgae it had never been previously tested for pathogen resistance promotion. As expected, Chl WT showed a very strong pathogen resistance promotion against PstT in NT.

The mutants LMJ.RY0402.200648 (CLiP mutant 200648/cLOG) and 2-LOG KOs (CRISPR/Cas9 mutants) have mutations in CK riboside 5’-monophosphate phosphoribohydrolase, the enzyme commonly known as LONELY GUY (LOG). LOG converts inactive CK nucleotides to the biologically active forms. It reacts specifically with CK nucleoside 5'-monophosphates, but not with

di-or triphosphate. This missing activation step could be the cause of the limited resistance promotion by these strains.

The difference observed in the protection against PstT between these two mutants was interesting since cLOG showed a resistance as good as the WT, while the 2-LOG KOs showed a significantly weaker protection than the WT. For the 2-LOG KOs, this hints that the mutation did indeed impair the algae to some extent from producing active CKs. However, since the cLOG mutant did as almost as well as the wild type, this produces a contradiction as far as the LOG gene is concerned, but it should not be the ultimate determinant because there was only one repetition with the cLOG mutants where the symptom development was as good as its wild type but the pathogen proliferation was significantly more than its wild type.

The experiments with the 2-LOG mutants produced reproducible and robust results (each mutant was independently tested two times for the symptom development and pathogen proliferation experiments). Hence, it can be concluded that a mutation in the LOG gene did in fact cause a slight disruption in the production of CKs and ultimately confer weaker resistance to the plant against pathogens.

LMJ.RY0402.45820 (HMBPP-KO) had a disruption in the HMBPP reductase enzyme synthesis. HMBPP reductase catalyses a step in the synthesis of DMAPP, the previously described precursor for most of the isoprenoid CKs. However, the MVA-pathway leading to the production of cZ was still active. Großkinsky and colleagues (Großkinsky et al. 2013) showed that cZ promoted much weaker defence responses in tobacco than tZ. And while there is interconversion between the *cis* and *trans* forms of Zeatin, the amounts are miniscule (Gajdošová et al. 2011). The disruption of this gene and the following experiment to evaluate the protection this strain would promote in NT showed that the HMBPP-KO strain was almost ineffective in inducing pathogen resistance. The differences between the HMBPP-KO and WT strain showed that with an impaired CK production the pathogen resistance promotion ability of Chl was impaired. This suggests that it was the CKs produced from the MEP-pathway in Chl that triggered the defence responses in the plant. To further confirm these results additional studies with Chl KOs of the CK biosynthesis pathway genes are required to gain a deeper understanding of the role of each gene in conferring the biocontrol characteristic to Chl.

5.4 Antimicrobial activity screening of leaves infiltrated with microalgae

Several previously published articles have shed light on the anti-bacterial and/or anti-fungal properties of microalgae. Most of these studies have been carried out using extracts obtained using chemicals or by directly mail-ordering compounds the different algae products (Hellio et al. 2000; Biondi et al. 2004). Subsequent studies have also been carried out using purified compounds from algae such as oligosaccharides and polysaccharides to infiltrate plant leaves and test their antimicrobial activity against pathogens (Klarzynski et al. 2003; Vera et al. 2011). However, no studies have been carried out to test the antimicrobial effect of live algae infiltrated into leaves against pathogens. Although it was expected that the leaf extracts with microalgae would form inhibition zones in the disk diffusion assays, that was not the case. It was also observed that even the positive controls (leaves fed with kinetin) did not form any inhibition zones in the disk diffusion assays. This can lead to the conclusion that there was something wrong in the extraction protocols itself, and perhaps the antimicrobial compounds lost their bioactivity after being plated on the disks, or the quantity of bioactive compounds was not high enough or concentrated enough.

The broth microdilution assays also proved unsuccessful to show any antimicrobial activity of the extracts. This assay uses a dye called INT to detect and quantify the respiratory activity in a solution. A colour change from colourless to pink implies that there is respiratory activity, and the darker the colour, the more respiratory activity there is and hence more organisms are present (Eloff 1998). The pathogen PstT failed to survive overnight in the 96-well plates, and this could be due to the reason that PstT is a gram negative bacteria, while this assay mostly works for gram positive bacteria (Klančnik et al. 2010).

As stated above, plants were first injected with microalgae and subsequently infected with PstT. In this sense, it is possible that CK does not interact directly with the pathogen in the plant but induces the immune responses of the plant. Many studies have established this property of beneficial microbes to upregulate defence responses of plants by either increased phytoalexin synthesis (Großkinsky et al. 2011), accumulating latent mitogen-activated protein kinases, chromatin modifications and changes in primary metabolism (Conrath 2011), jasmonate, ethylene or salicylic acid dependent signalling pathways (Wees, der Ent, Pieterse 2008), phosphoinositide- and ABA-dependent signalling components, NPR1-dependent signalling pathways (der Ent et al. 2009) etc. This shows that priming is dependent on many intricate and interconnected pathways, and it is also dependent on

both the pathogen and the agent which induces the priming responses (der Ent et al. 2009). Hence, perhaps a simple antimicrobial activity screening with the leaf extracts is not enough to give further proof of concept of the pathogen resistance promotion properties of Chl, but more complicated studies such as phytohormone profiling or RNASeq of the WT versus mutants is needed.

5.5 Algae as a bio-stimulant

The Chl strains have proved to promote growth in three individual experiments in tomato, tobacco and *Arabidopsis*; at seedling stages for tobacco and *Arabidopsis*, and for seed germination for tomato seeds. As Chl is already established to be a microalga with a high production of CKs, it can be assumed that this growth promoting ability is mainly due to the CKs.

Many reports have previously shown that algae or algae extracts can contribute to a faster germination of tomato (Garcia-gonzalez, Sommerfeld 2016). However, it had not been previously shown to be the case for Chl. Experiments with microalgae-induced seed germination clearly showed faster seed germination due to Chl mutants and wild types. As previously mentioned, the algae produce a wide range of phytohormones, many of them are growth hormones, which could trigger a faster germination. In addition, the algae itself could serve as a bio-fertilizer, providing nutrients to the seeds and thereby promote a faster growth.

What was interesting to observe in the seed germination experiments is that the supernatant from the CRISPR/Cas9 mutants PH 144 and PH 147 promoted the faster and higher percentage of seed germination. This could lead us to consider that there are compounds secreted by the algae which stay in the supernatant that can positively promote growth of seeds. Also, the tomato seeds treated with the CRISPR/Cas9 mutant had the highest germination percentages in comparison to the other treatments. It is already quite clear from the biocontrol experiments in NT that the production of CKs in these mutants is lower than in the WT. This could mean that a high production of CK in algae can be growth inhibitory for seed germination. This is in accordance with the experiment by Garcia-gonzalez & Sommerfeld (2016) where a high concentration of algae extracts were inhibitory for the growth of tomato, and also that possibly a high production of CKs negatively affects the growth of tomato, such as high amount of CKs inhibit growth of barley (Kirschner et al. 2018). Since the seeds treated with cLOG and HMBPP KO mutants also had germination percentages lower or equal to the germination percentage of the seeds treated with the WT, it probably means that there is an optimum concentration of cytokinins or algae that boosts seed germination.

Chl not only promoted seed germination, but the WT strain was also shown to promote the growth of tobacco and *Arabidopsis* in the seedling stage in two independent experiments. This is conclusive proof that Chl indeed is a promoter of the growth of plants and is hence a very effective bio-stimulant, which was not surprising due to the high production of CKs and the long history of the use of microalgae as bio-fertilisers in agriculture (Babu et al. 2015; Garcia-gonzalez, Sommerfeld 2016; Win et al. 2018; Kholssi et al. 2019).

The live microalgae solutions in both experiments with tobacco and tomato were exhibited to improve the growth of the plants, which could imply that microalgae when administered live have the best effect on the plants, since the biocontrol capability of the live solutions proved to be better than the extract.

It should be mentioned that although there was no significant growth in tomato seeds, this was probably due to a problem with the seeds since they were variable in size on the day of inoculation, so the results obtained were unreliable. Another fascinating observation was the influence of live algae on the SWC of the soils in which the *Arabidopsis* grew. The CC 125 live treated soils in well-watered plants had a significantly higher SWC than the other soils, which shows that it had the highest water retention capability, probably due to the fast growth of the plants in it. While further and more specific studies need to be carried out in order to confirm this hypothesis, these are promising evidences.

5.6 Role of Chl in response to abiotic stress in plants

In the drought tolerance experiment, the weights of the drought treated plants were too variable and not significant to come to any conclusive results. However, the survival of five out of 36 plants of the CC 125 culture treated plants (compared to only one surviving in the control and zero surviving of the other two treatments) in the *Arabidopsis* drought tolerance experiment is preliminary proof in conjecture with other independent experiments on tomato and tobacco which prove that CC 125 indeed has a positive effect on growth, and hence may also provide some sort of abiotic stress tolerance to plants. A limitation of this experiment was that the drought condition was too prolonged due to which almost all the plants died, hence this experiment needs to be repeated with a shorter drought period in order to reach to a proper conclusion.

It is not too far-fetched to assume that Chl would provide tolerance to abiotic stress conditions in plants, as Chl is a high CK-producing organism and there have been many studies which have proved

that cytokinins are essential in promoting tolerance towards abiotic stress conditions for plants (Tran et al. 2007; Rivero et al. 2009; O'Brien, Benkova 2013; Zwack, Rashotte 2015). Moreover, there have been studies which have showed that CKs improve the drought tolerance resistance of rice (Peleg et al. 2011; Reguera et al. 2013). There have been no studies till date which have established the role of microalgae as a promoter of abiotic stress tolerance in plants, although there have been studies to support the role of microalgae as a model for abiotic stress tolerance itself (Holzinger, Pichrtová 2016). Hence, keeping all these previous studies and the high CK-producing capability of Chl in mind, it can very well be possible that Chl is a good promoter of abiotic stress tolerance in plants, and this experiment needs to be repeated with the mutants in order to get more insight and conclusive evidence regarding this idea. Also, more experiments with other abiotic stresses such as flooding, metal toxicity, extreme temperature changes, salinity etc. can be carried out to see if Chl can provide the plant with tolerating capabilities.

5.7 Further perspectives with Chl

Various microalgae species have already been established to provide multiple benefits to the soil and plants, such as improvement in seed germination, enhanced growth of plants, improved nutritional characteristics, enhanced soil microbial activity, improvement in soil organic carbon etc. (Renuka et al. 2018). In the experiments performed in this thesis, the growth stimulation properties, abiotic stress responses and biocontrol capability of Chl has been established via many independent experiments with tobacco, tomato and *Arabidopsis* (Figure 5.1). This proves that Chl has excellent potential to be an environmentally sustainable multi-purpose biocontrol agent. Such sustainable options are a dire need right now in agriculture, as conventional pesticides are detrimental to the environment since they negatively affect the populations of important pollinators such as bees (Gill, Ramos-Rodriguez, Raine 2012). However, despite the increase use of pesticide, crop losses have remained the same in the past 40 years (Oerke 2006). Also, food production needs to double by 2050 in order to sustain the increasing population, but the current agricultural practices are not able to achieve that (Ray et al. 2013). Keeping all these factors in mind, focus has shifted to "green" bio-fertilisers and biocontrol agents. Chl is a very good contender for that, and Chl-based environmentally sustainable biocontrol agents and bio-fertilisers could be a promising option for the future of agriculture.

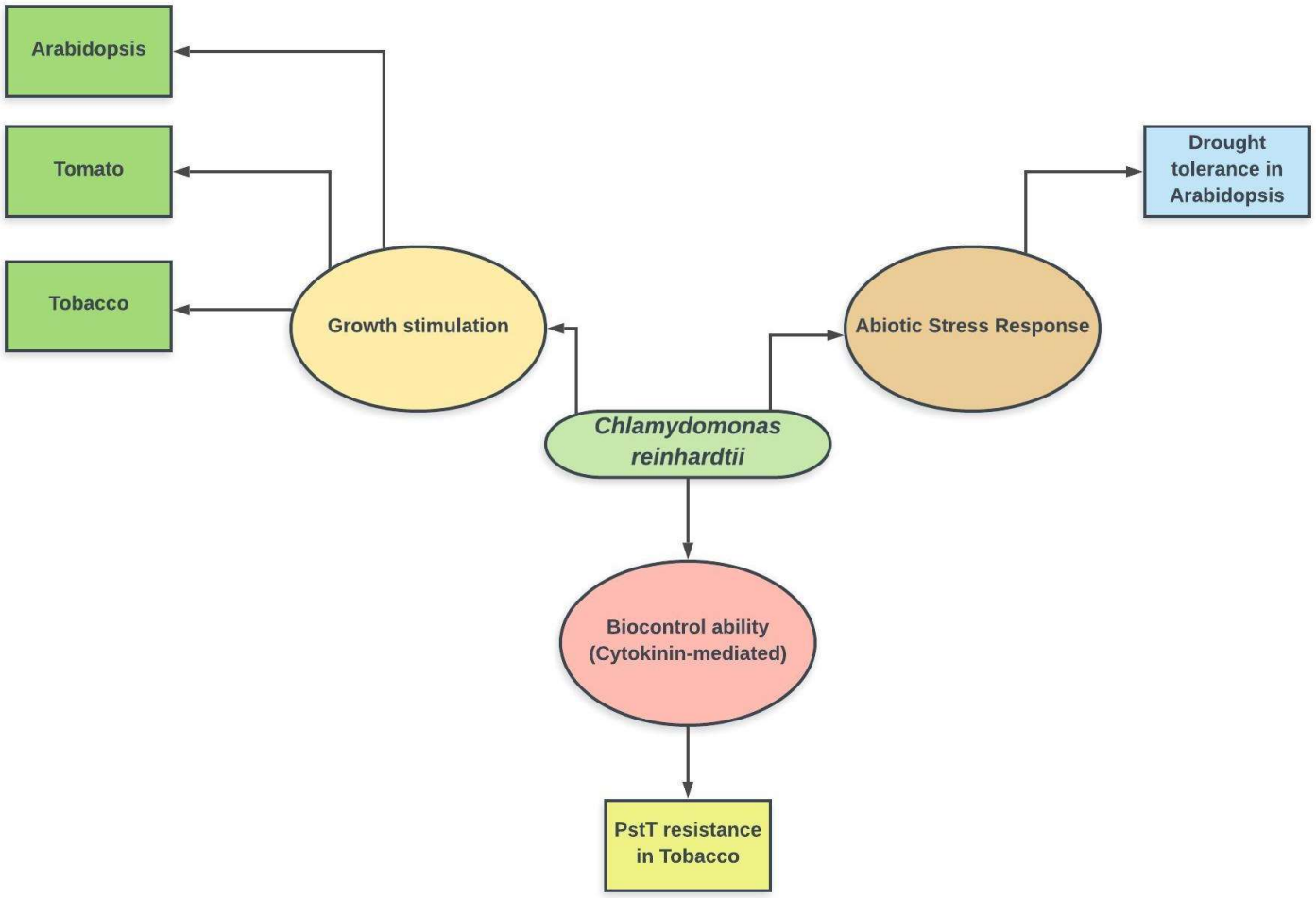


Figure 5.1: The combined growth stimulation, abiotic stress tolerance promoter and biocontrol capability of *Chlamydomonas reinhardtii* make it an excellent candidate for environmentally sustainable multi-purpose biocontrol agents for the future in agriculture.

6. Conclusion

In conclusion, *Chlamydomonas reinhardtii* does indeed provide resistance to *Pseudomonas syringae* pv. *tabaci* in tobacco, and this resistance is cytokinin-dependent.

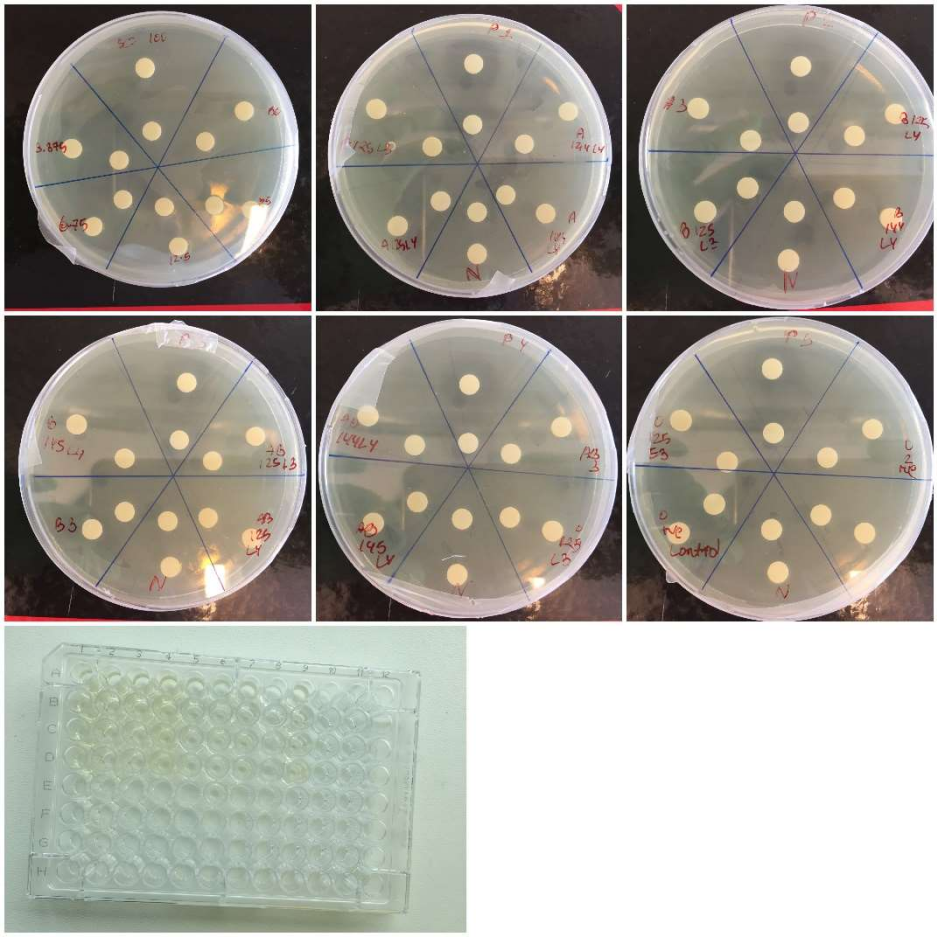
This has been possible to understand due to the use of knockout mutants of the LOG and HMBPP reductase enzyme gene, which are two genes in the cytokinin biosynthesis pathway. The HMBPP reductase gene seemed to be more important in providing resistance to the plants than the LOG gene.

Also, *Chlamydomonas reinhardtii* has proven to stimulate growth in tomato, tobacco and *Arabidopsis* in their seedling and seed stages of development.

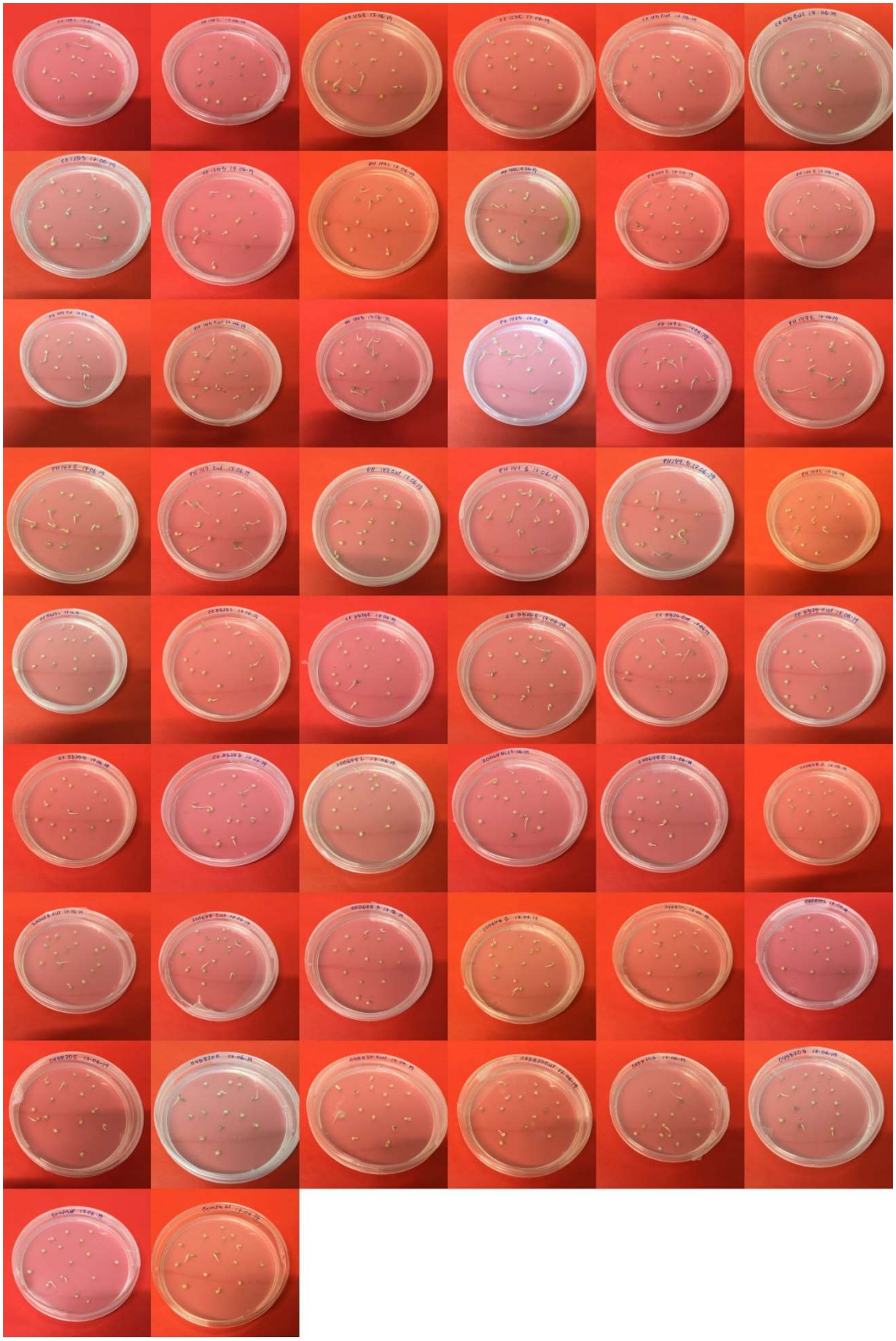
Last, but not the least, this microalga shows promise to promote drought tolerance in *Arabidopsis*.

A combination of all these characteristics make this microalga a promising contender to make bio-control agents which have a multi-purpose feature. In the past decades, various microalgae have been shown to have many benefits to the soil, water and the environment (Renuka et al. 2018; Rizwan et al. 2018; Win et al. 2018). However, there were no studies with *Chlamydomonas reinhardtii* to explore these aspects of plant resistance promotion in combination with its bio-fertilising capabilities, despite it being a model organism for many studies and having a high production of CKs.
This study has showed tremendous potential for *Chlamydomonas reinhardtii* as a biocontrol agent and a bio-stimulant for plant species, as it contains characteristics due to which environmentally sustainable multi-functional biocontrol agents can be developed from it.

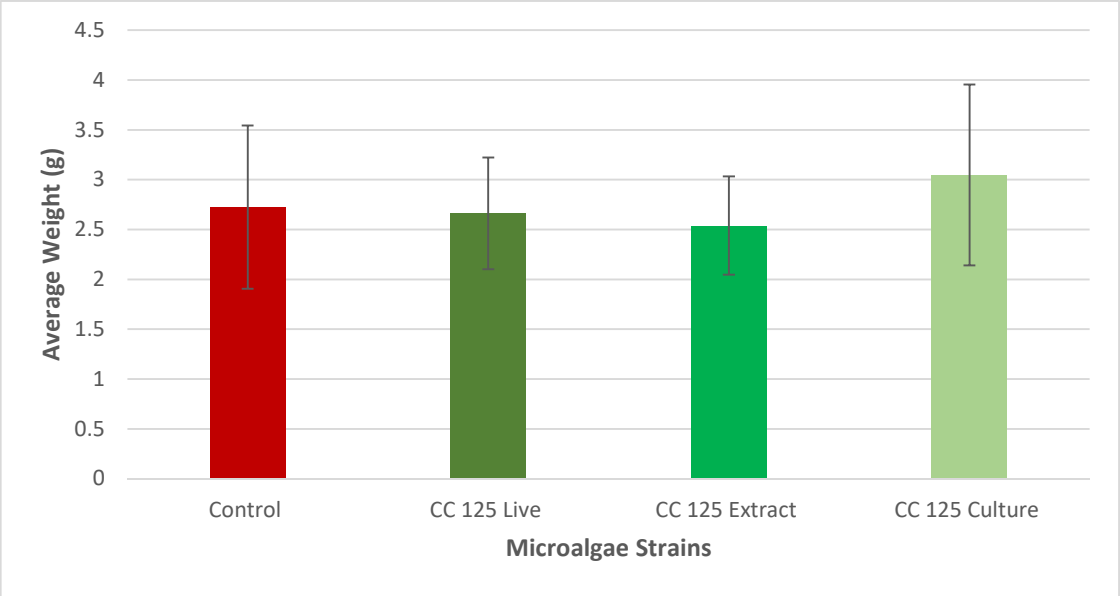
7. Appendix



Appendix 1 – **A1**: Examples of antimicrobial activity results with disk diffusion assays (Top and middle row), and broth microdilution assays (bottom row), with no antimicrobial activity being exhibited against PstT.



Appendix 2 – **A2**: Pictures of all the Petri dishes of all the treatments of the algal promoted seed germination experiments.



Appendix 3 – **A3**: Comparison of the average weights of tomato plants inoculated with different treatments of CC 125 solutions at their seedling stage. The weights of the plants in each treatments are not significantly different from each other (Unpaired Student's t-test, $p > 0.05$, $n=10$).

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