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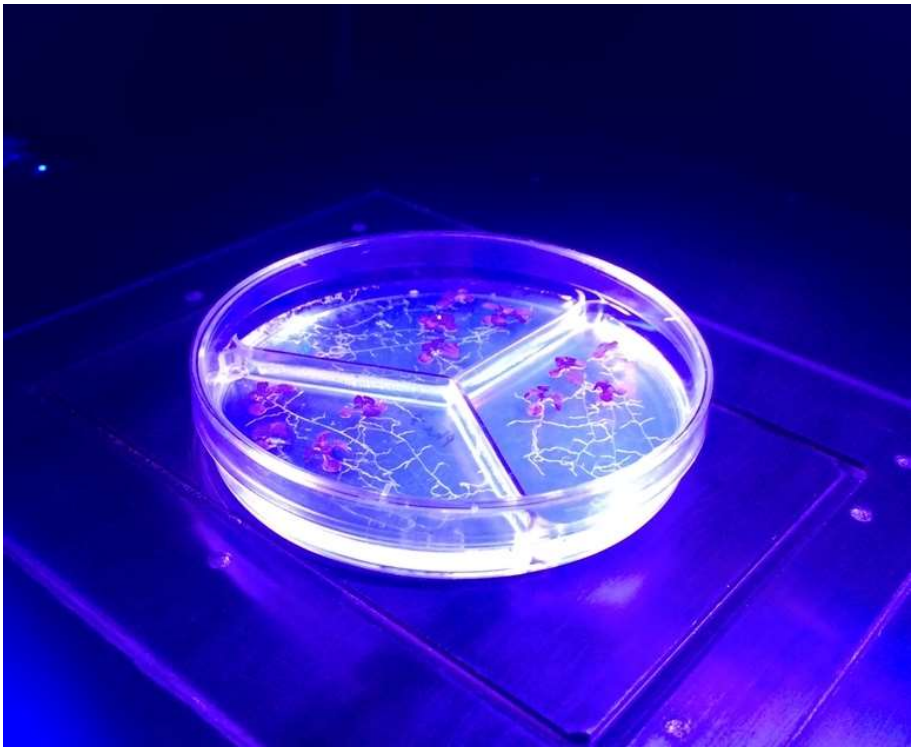

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Abstract

Circadian rhythms are daily rhythms in gene expression and physiology with a period of approximately 24 hours. Despite the knowledge of circadian rhythms in animals, plants, fungi and cyanobacteria, the circadian biology of one of the most economically important groups of organisms remains almost completely unstudied: rhizobacteria. PAS domains are found in circadian clock components in a number of kingdoms of life. Several PAS domain-coding genes exist in the rhizobacterium *Bacillus* subtilis, with *ytvA* being one and also a blue light photoreceptor. Here I used bioluminescence timecourse imaging experiments to measure *ytvA* promoter activity in transgenic *B. subtilis,* following inoculation onto the roots of plants entrained to opposite light regimes. Results showed *ytvA* promoter activity oscillated with a period of approximately 24 h, indicating that circadian rhythms in *Arabidopsis* host plants may provide cues to entrain *B. subtilis* cultures. In experiments investigating the influence of oppositely-entrained *B. subtilis* on expression of the *Arabidopsis* clock gene *CCA1,* I found cultures lit during the day lengthened *CCA1* period and cultures lit during the night shortened *CCA1* period. This may suggest *B. subtilis* can in turn affect plant circadian clock function. This preliminary study provides the first evidence for the potential bidirectional signalling of circadian timing information between plants and bacteria, building on previous work demonstrating the effects of the rhizosphere on plant circadian clock function. Future work should seek to refine bacterial entrainment regimes, uncover bacterial clock genes and pinpoint entrainment signals involved in this interaction. Studying rhythmicity in rhizobacteria may also require the use of complex intact soil microbiomes instead of single-species experiments. Understanding the functioning of bacterial circadian clocks and their interactions with host plants has implications for the use of bacteria in industry, the treatment of microbe-associated diseases and the use of rhizobacteria in agriculture.

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Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED : .....  ..... DATE: 15/04/19

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List of Abbreviations

Abbreviation	Definition
°C	Degrees Celsius
µl	Microlitres
CCA1	CIRCADIAN CLOCK-ASSOCIATED 1
Cm	Chloramphenicol
CV	Crystal violet
DET1	DEETIOLATED 1
ELF	EARLY FLOWERING
g	Grams
GI	GIGANTEA
h	Hours
L	Litre
LB	Lysogeny broth
LD	light:dark
LL	light:light (i.e. constant light)
LHY	LATE ELONGATED HYPOCOTYL
M	Molar
ml	Millilitre
mM	Micromolar
mm	Millimetre
MS	Murashige & Skoog
MST	Murashige & Skoog media supplemented with 1% tryptone
PGPR	Plant growth-promoting rhizobacteria
pH	Potential hydrogen
PRR	PSEUDO-RESPONSE REGULATOR
rpm	Revolutions per minute
s	Seconds
spp.	Species
TOC1	TIMING OF CAB EXPRESSION1
w/v	Mass/volume percentage
ZTL	ZEITLUPE

Chapter 1: Introduction

Life on earth is biologically stressful: the rotation of the planet generates daily changes in light, temperature and humidity to which organisms must adjust. The cyclical nature of these changes makes them somewhat predictable and thus organisms have evolved an internal system known as the circadian clock to accurately time their responses. This endogenous timekeeper co-ordinates an organism's physiological processes with their environment and in doing so enhances their survival (Ouyang et al. 1998; Green et al. 2002; Woelfle et al. 2004; Dodd et al. 2005). The timing of biological processes is easily observed across the tree of life, from early documentations of rhythmic leaf movements in mimosa in 1729, asexual spore cycles in fungi, and daily patterns of wheel-running activity in rodents, to more recent studies comparing temporal changes in whole genomes (de Mairan 1729; Pittendrigh & Daan 1976; Doherty & Kay 2010). However, the circadian biology of non-photosynthetic bacteria, one of the most ubiquitous groups of organisms, remains substantially understudied. Here I focus on one of the most economically important groups of bacteria, rhizobacteria.

Root-colonising bacteria (rhizobacteria) have long been recognised as beneficial for both the production of industrially-relevant compounds and for enhancing plant growth and suppressing disease in agriculture (Vejan et al. 2016). In industry, isolated bacteria are used in a wide range of sectors including food production, textile processing, and manufacturing of products such as organic acids, enzymes and pharmaceuticals (Singh et al. 2016). Many of these important rhizobacteria are found in the genus *Bacillus* (van Dijl & Hecker 2013; Lyngwi & Joshi 2014). Despite their economic value, there remains much to be discovered about their circadian biology and how they may influence the circadian clock and subsequent fitness of host plants. In this chapter, I will focus on the current literature on plant and bacterial circadian biology and detail the importance of plant-colonising *B. subtilis* for both industry and agriculture.

1.1. Circadian rhythms

1.1.1. The circadian clock: a fitness advantage for plants

The circadian oscillator generates circadian rhythms that are daily rhythms in gene expression with a characteristic period of approximately 24 hours. This helps organisms anticipate changes in the external environment and coordinate their metabolism and biochemistry to these changes. In addition to a period of 24 hours, circadian rhythms are entrainable by exposure to external stimuli such as light and temperature, persist in the absence of environmental cues and maintain a

relatively constant period in the face of changing external temperatures (known as temperature compensation) (Pittendrigh 1960).

Circadian systems are common to both unicellular and multicellular organisms, and have been well-studied in cyanobacteria, plants, fungi, flies and mammals. The circadian system in plants is often described as being composed of three major parts: the circadian oscillator (known also as the circadian clock), input pathways which entrain the clock with external cues, and output pathways to control physiological and metabolic pathways in the plant, such as leaf movement, hypocotyl elongation, stomatal opening and photoperiodic flowering. Inputs that synchronise the plant circadian clock to the external environment are known as zeitgebers and include stimuli such as light, temperature and sugars. Synchronising internal processes to the changing external environment provides a fitness advantage to plants. Various studies have compared the fitness of plants lacking functioning circadian oscillators to their wild-type counterparts. Plants overexpressing certain circadian genes are less viable under very short-day conditions and flower later than plants with functioning oscillators, a change which could affect the reproductive success of plants in their native habitat (Green et al. 2002). A later study by Dodd et al. (2005) demonstrated that when the circadian clock period matches that of the external environment, plants fixed more carbon, contained more chlorophyll, had higher vegetative biomass and survived better. This fitness advantage is likely due to the correct anticipation of dawn and dusk, with the associated stomatal opening and synthesis of light-harvesting proteins which is under circadian control (Harmer et al. 2000).

1.1.2. A complex plant system: interlocking transcription-translation feedback loops

The circadian clock in the model plant *Arabidopsis thaliana* is composed of a complex network of over 20 genes which form interlocking transcription-translation feedback loops (Hsu & Harmer 2014) (Figure 1.1.). This means different clock proteins are active at different points in the day and night, and reciprocally regulate each other. These components can be partially divided into those whose activity peaks in the morning, termed morning-phased components, a later peaking group known as day-phased components, and finally evening-phased components.

Morning-phased components include CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) which are highly conserved MYB-like transcription factors whose transcripts peak after dawn and are known to function synergistically, binding to the same promoter region of a protein known as the chlorophyll A-B binding protein (CAB) (Schaffer et al. 1998; Wang & Tobin 1998; Lu et al. 2009)

As part of the reciprocal behaviours of the circadian oscillator, these proteins repress expression of evening components such as *TIMING OF CAB EXPRESSION 1* (*TOC1*, also known as *PRR1*), a process which requires the photomorphogenesis component DEETIOLATED 1 (DET1) (Millar et al. 1995a; Lau et al. 2011). Conversely, TOC1 can repress expression of *CCA1* and *LHY,* thus forming a transcriptional feedback loop (Gendron et al. 2012). Alterations to rhythms in mutant plants can be investigated by either measuring cotyledon leaf movement, or by using a light-emitting luciferase (*luc*) reporter attached to the promoter of circadian genes and measuring light output from the plant over time. Using these methods, it was found that loss-of-function *toc1-1* mutants exhibit a shortened period of approximately 21 hours under constant light, as do *cca1* and *lhy* mutants, although the latter pair are partially functionally redundant (Millar et a. 1995; Green & Tobin 1999; Mizoguchi et al. 2002). These significant effects on the plant circadian rhythm demonstrates the importance of these 3 core clock components on generation of functioning circadian rhythms.

As part of the complex genetic interactions within the circadian clock, CCA1/LHY can repress other evening genes such as *GIGANTEA* (*GI*), *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3 (ELF3)* and *EARLY FLOWERING 4* (*ELF4*). Day-phased components like PSEUDO-RESPONSE REGULATORS 9, 7, and 5 (PRR9, PRR7, PRR5) act later to repress *CCA1/LHY,* incorporating them into the reciprocal feedback loops (Nakamichi et al. 2010). The PRR components are expressed through the day in the following order with approximately 2-3h intervals between peak expression: *PRR9* after dawn*, PRR7, PRR5, PRR3* and *TOC1* (*PRR1*) in the evening (Matsushika et al. 2000). Together, this repression of *CCA1/LHY* by PRR5, PRR7, PRR9 and TOC1 ensures *CCA1/LHY* is only expressed for a limited time in the morning.

Another important group of clock components in *Arabidopsis* are the *REVEILLE* (*RVE*) MYB transcription factors, including *RVE8* (also known as *LCL5*), *RVE4* and *RVE6.* Like *CCA1/LHY*, *RVE8* has morning-phased expression and regulates the expression of *TOC1* in the subjective afternoon by binding to the evening element (EE) in the *TOC1* promoter. However, RVE8 activates *TOC1* as opposed to repression through CCA1/LHY which is thought to fine-tune the expression waveform for *TOC1*, increasing its expression in the evening (Farinas & Mas 2011). In fact, RVE8 induces the expression of many other evening-phased genes with EEs including *PRR5,* but is then repressed by PRR5, PRR7 and PRR9, forming another negative feedback loop (Rawat et al. 2011). The two other *RVE* genes, *RVE4* and *RVE6,* are partially redundant with *RVE8.* However, loss-of-function mutants in all 3 genes results in reduced transcription levels of evening clock genes and a phase delay in several clock genes (Hsu et al. 2013). It should be noted that the EE is a vital part of the evening regulated genes in the circadian clock, existing in the promoters of most evening-phased clock genes such *TOC1, PRR5, GI, LUX* and *ELF4* (Covington et al. 2008; Harmer et al. 2000). Finally, at the end of the

day three additional components - LUX, ELF3 and ELF4 – interact to form the "evening complex" which represses *PRR9* (Helfer et al. 2011; Nusinow et al. 2011). Loss of any of the evening complex genes results in an arrhythmic phenotype under constant conditions (Hicks et al. 1996; Hazen et al. 2005; McWatters et al. 2007).

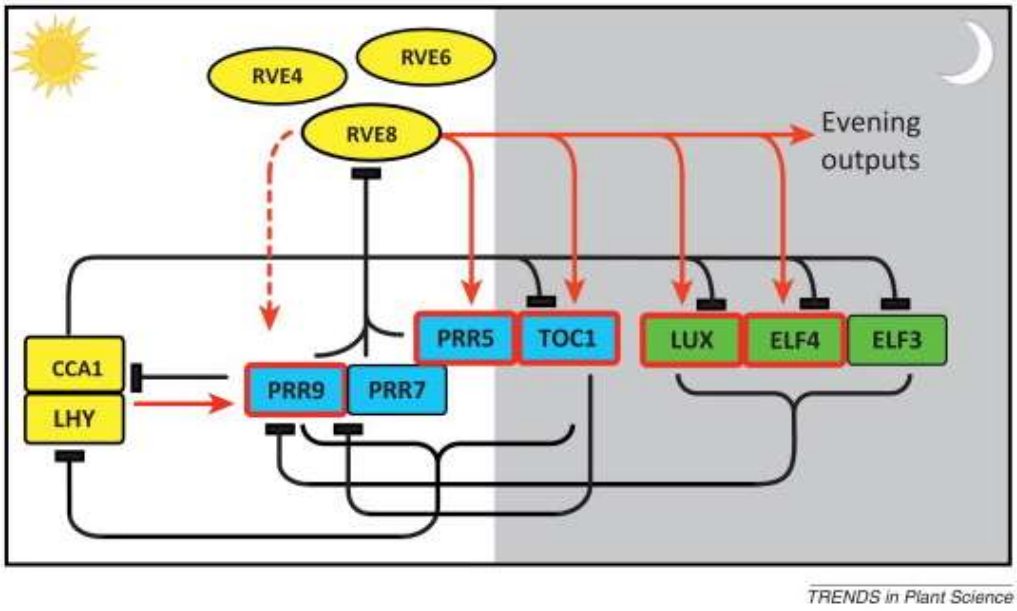

Figure 1.1. Simplified model for the transcriptional regulation of the circadian clock in *Arabidopsis thaliana.* The morning components CIRCADIAN-CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and the afternoon REVEILLE (REV) components (RVE4, RVE6, RVE8) are shown in yellow. Coloured blue are the PSEUDO-RESPONSE REGULATOR components (PRR9, PRR7, PRR5) and TIMING OF CAB EXPRESSION 1 (TOC1, also known as PRR1). Other evening components including LUX ARRHYTHMO (LUX), and EARLY FLOWERING 3 and 4 (ELF3, ELF4) are shown in green. Components with the evening-element (EE) in their promoter regions are bordered red. (Reproduced from Hsu & Harmer 2014).

Several post-transcriptional and post-translational mechanisms also modulate circadian function. This includes pre-mRNA processing (5' capping, splicing and 3' poly-adenylation). Multiple clock genes (including *CCA1, LHY, PRR9, PRR5, PRR3, TOC1, RVE4,* and *RVE8*) undergo alternative splicing. For *PRR9,* this is regulated by PROTEIN ARGININE METHYLTRANSFERASE (PRMT5), and the spliceosome component SNW/Ski-interacting protein (SKIP) which additionally controls alternative splicing of *PRR7, CCA1, LHY* and *TOC1* (Sanchez et al. 2010; Wang et al. 2012). Following translation, interaction of clock proteins with other proteins can alter their stability, such as the ubiquitination and subsequent degradation of TOC1 and PRR5 by the F-box protein ZEITLUPE (ZTL) (Somers et al. 1998; Más et al. 2003a).

1.1.3. Cell-specific and tissue-specific plant circadian clocks

Another layer of complexity in the circadian system arises from differences in the functioning of the circadian oscillator between different cells and tissues. For example, Thain et al. (2000) used local light treatments on different parts of intact plants to test for de-synchronisation. Tobacco (*Nicotiana tabacum*) seedlings were grown under light-dark cycles (LD) before transfer to constant light (LL) where the cotyledons were alternatively covered with opaque foil to put the cotyledon rhythms in antiphase with each other (12 hours apart). Rhythms were studied in the plants using a *luc* reporter attached to the promoter of 3 circadian-regulated genes: phytochrome B1 (*PHYB*), chalcone synthase (*CHS*) and chlorophyll a/b-binding protein (*CAB2*). All oppositely entrained cotyledons retained their antiphase rhythms under LL, indicating rhythms in the leaves are autonomous.

There is also inter-organ variability in circadian clocks. James et al. (2008) analysed differences in the circadian clock between shoots and roots in *Arabidopsis*, using clock gene transcripts to measure oscillations in the plants following transfer to LL from 12 h LD cycles. Interestingly, analysis of the *CCA1/LHY* transcript revealed that the period was 2 hours longer in the roots than the shoots. *PRR7* and *PRR9* also had rhythmic expression in both shoots and roots, but again the period was longer in the roots. In addition, many of the genes that oscillated in the shoots, such as *GI, LUX, ELF3, ELF4, PRR3, PRR5* and *TOC1,* did not oscillate in the roots and instead retained consistently higher transcript abundance. This study indicates that the clock in the roots only operates the morning phase loop, and evening genes, such as *TOC1*, do not contribute to the root clock. The lack of rhythmic evening gene expression was, at least in part, thought to be due to the failure of the LHY protein to cause EE-mediated inhibition of gene expression in the roots. The peak abundance for *CCA1* and *LHY* transcripts was slightly delayed in roots compared to shoots, which may indicate that a timing signal, such as photosynthetic sugars, may be transmitted from shoots to roots. A more recent study by Bordage at al. (2016) developed an imaging system to monitor rhythms in shoots and roots separately and found that morning and evening genes were rhythmic in both organs, in contrast to the findings by James et al. (2008). The *toc1-4* mutant shortened the period in both the shoot and root clocks by 3-4 h, supporting the evidence that *TOC1* plays an important role in root clock function. Some findings did however match, including a longer circadian period in the roots than the shoots. Results also showed that the root clock could be independently entrained to low levels of light in LD cycles, even in antiphase to shoot illumination. Additional experiments showed that sucrose did not affect the difference in period between the shoot and root clocks. Together, these results confirm the roots are directly entrained by light signals and use light signals preferentially over sucrose signals, in contrast to the suggestion by James et al. (2008) that the roots are entrain by photosynthetic sugar signals from the shoots.

The differences between root and shoot clocks is thus likely due to organ specific light inputs. Nimmo (2018) followed up on this by investigating responses of the root and shoot clocks to different quality light inputs (by varying the intensity of red and blue light). The period of the root clock was again longer in roots than shoots. Additionally, the root clock was more sensitive to red light than blue light, as shown by period shortening with increasing red light than increasing blue light. Decapitation experiments, where the shoot was removed but part of the root remain exposed to light, demonstrated that these shortening effects could be transmitted down the root from the root tissue exposed to red light to the darkened section. This evidence supports the notion of entrainment of root tissue via light piping from tissues exposed to light and reflects the physiological conditions of the root embedded in the soil. Since the shoot tissue was removed, entrainment via light piping instead of sucrose signalling from photosynthetic tissues appears primarily responsible for shoot-root synchronisation in dark grown roots.

At a smaller scale, differences between tissues have been noted. Coupling of circadian rhythms between leaf tissues was identified by Endo et al. (2014), who used microarray analysis to analyse diurnal patterns of gene expression in mesophyll, vasculature and epidermis. About 50% of the leaf genes had oscillatory expression, however only 10.5% cycled together, suggesting tissue-specific and day-length specific regulation. In the same study, Endo et al. developed a tissue-specific luciferase assay (TSLA) to measure circadian rhythms independently in the 3 different leaf tissues and found the vasculature clock was able to regulate the circadian clock in neighbouring mesophyll cells. This suggests that the different tissues have distinct circadian clocks but there is some coupling between them.

There is also evidence for differences in circadian function at the intercellular level. Under constant light, cells in the leaf become desynchronised with each other, giving different spatiotemporal patterns of peak *CCA1::LUC* expression across the leaf. This desynchronization indicates there is weak coupling between the cells, but they can be resynchronised when placed under LD cycles again (Wenden et al. 2012). Spatiotemporal patterns of circadian gene expression have also been reported in roots: Fukuda et al. (2012) reported a stripe wave pattern of circadian *CCA1* gene expression which originated from cells at the root tip. Every section of the root exhibited self-sustained oscillations but coupling between cells remained.

1.1.4. Circadian rhythms in other organisms: cyanobacteria

Circadian clocks have also been rigorously studied in non-eukaryotic organisms. The model cyanobacterium *Synechococcus elongatus* has a circadian clock that is arguably the best understood of all circadian systems. Rather than the transcription-translation feedback loops in the plant system,

the core oscillator operates through post-translational modification using phosphorylation cycles. The core oscillator is composed of 3 proteins: KaiA, KaiB and KaiC. Of these, KaiC is the key component and the level of its phosphorylation at serine 431 and threonine 432 residues provides a marker of circadian phase (Nishiwaki et al. 2004). The level of phosphorylation regulates the association and disassociation with the KaiA and KaiB components and controls the switching of KaiC between its autokinase and autophosphatase states (Nishiwaki et al. 2007; Rust et al. 2007) (Figure 1.2.). Output signals are transmitted via a two-component signalling pathway comprised of the histidine kinase SasA and the cognate response regulator RpaA to drive rhythmic patterns of gene expression. RpaA is the primary factor in controlling these rhythms, evidence of which can be seen in that loss of *sasA* results in altered rhythmicity, whereas loss of *rpaA* results in completely absent rhythmic gene expression (Takai et al. 2006).

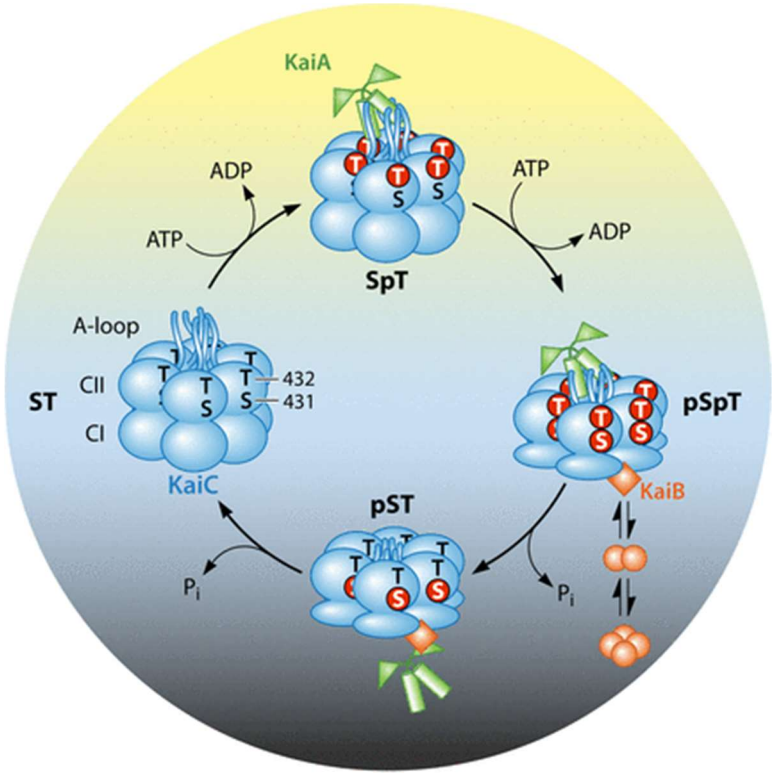

Figure 1.2. Basic mechanism of the cyanobacterial circadian oscillator. KaiC (blue) formed of 2 rings (CI and CII), is the core component. During the day, KaiA can bind to the A loop, resulting in phosphorylation of KaiC on its serine 431 and threonine 432 residues. This promotes interaction of KaiB with KaiC, and subsequent loss of KaiA. Finally, this results in the activation of KaiC autophosphatase activity and dephosphorylation of T432 and S431 during the night. (Reproduced from Cohen & Golden 2015).

Further work by Markson et al. (2013) provided the underlying mechanism for this control of gene expression. In its phosphorylated state (as a result of phosphotransfer from SasA), RpaA is active as a DNA-binding transcription factor, binding to 110 sites and activating global regulators such as

bacterial sigma factors which play critical roles in transcription activation. Alongside global gene expression patterns, this circadian system also controls the timing of cell division and degree of chromosome compaction.

The cyanobacterial clock is not entrained by stimulation of photoreceptors, but rather through sensing the redox state of the cell, which changes with photosynthetic activity. The protein CikA, together with KaiA, can bind to the oxidised form of quinone which increases at the onset of darkness. This binding to quinone appears to result in aggregation and degradation of CikA and KaiA, suggesting these redox state-dependent changes in the concentrations of these components control the synchronisation of the clock with daily light-dark cycles (Ivleva et al. 2006; Kim et al. 2012). Additionally, other photosynthetic metabolites provide inputs, such as changes in the ATP/ADP ratio which is sensed by KaiC (Rust et al. 2011).

1.1.5. Circadian rhythms in non-photosynthetic bacteria

Although circadian clocks have been well studied in cyanobacteria, there are few reports of circadian oscillations in non-photosynthetic bacteria. Before 1985, circadian rhythms were thought not to exist within prokaryotes because they were regarded as too 'simple' and an endogenous timekeeper with a period of 24h was thought to be disadvantageous; since bacteria divide multiple times in 24h, a dogma known as the "circadian-infradian rule" (Ehret & Wille 1970; Edmunds 1983). Additionally, there was an argument that the need for bacteria to quickly respond to external stimuli under stress situations or when nutrients suddenly became available would not match well with temporal circadian control. However, this view was later counteracted by the fact cyanobacteria are also simple organisms with a cell division time of less than 24 h, and they possess a circadian clock that does not disrupt their ability to exhibit stress responses if the external environment changes rapidly (Globbelaar et al. 1986; Johnson 2004).

Phylogenetic studies by Dvornyk et al. (2003) and Loza-Correa et al. (2010), who searched for homologues of *kai* genes in other bacteria, paved the way for circadian control in non-photosynthetic bacteria. They found *kaiA* is only present in cyanobacteria, but homologues of *kaiB* and *kaiC* are shown throughout Archaea and Bacteria. In Archaea, *kaiC* homologues are found in almost all major taxa, whereas only in 3 other taxa in Bacteria (Proteobacteria, Thermotogae and Chloroflexi). Interestingly, Proteobacteria includes several species which form close associations with organisms with identified circadian rhythms. One such example is the nitrogen-fixing rhizobacterium *Sinorhizobium medicae* which possesses a *kaiC* homologue. This species forms root nodules on plants in the *Medicago* genus (Rome et al. 1996). Hypothetically, aspects of circadian rhythmicity that match that of the host plant could be beneficial in that it would allow the bacteria to anticipate

the secretion of photosynthetically-associated plant exudates, thus switching on the genes for metabolising these products at the correct time. Another proteobacterium is *Pseudomonas putida,* which can form associations with plants, subsequently receiving nutrients in return for providing a protective role to the host against pathogens (Molina et al. 2000; Espinosa-Urgel et al. 2002; Bernal et al. 2017). Genetic analysis has confirmed the metabolic ability of the bacterium for digesting plant exudates: Nelson et al. (2002) found genes for an opine transporter and enzymes for metabolic pathways involved in breaking down a number of plant derived opines. *P. putida* also has a *kaiC* homologue and once again would benefit from the anticipation of the secretion of plant nutrients.

In addition to the phylogenetic analyses by Dvornyk et al. (2003) and Loza-Correa et al. (2010) on *kai* gene homologues, diurnal variations in bacterial growth and mobility have been investigated as a measure of circadian outputs. The human gastrointestinal system exhibits circadian patterns of gene expression and diurnal variations in motility and secretion. *Enterobacter aerogenes* isolated from the human gut is sensitive to gut secretions of melatonin and exhibits circadian patterns of swarming that are temperature-compensated, suggesting a circadian clock (Paulose et al. 2016). In terms of plant associations, early studies have shown short-term fluctuations in microbial species composition at the community scale, including diurnal fluctuations in bacterial numbers of *Erwinia* in the phyllosphere of sugar beet (*Beta vulgaris*) (Thompson et al. 1995). As aforementioned, *P. putida* has a *kaiC* homologue and interestingly, circadian variations in growth pattern have been reported for this species. Soriano et al. (2010) grew plates of *P. putida* on solid media supplemented with dye under LD cycles of 16 h/8 h and measured growth rings of different colour intensity. The successive rounds of light and dark rings developed with a period of approximately 24 h, and this periodicity could be maintained for a further 2 days following transferral to constant light conditions. Since changes in light are one of the signals for plant entrainment, it is easy to envisage how they could provide a useful entrainment signal for plant associated bacteria to prepare for photosynthetic products from the plant. Together, these studies provide the first evidence towards circadian systems in non-photosynthetic bacteria.

Conversely, alterations to the plant circadian clock have been shown to have temporal effects on the rhizosphere community, suggesting signalling of circadian timing information from plants to bacteria. For example, Hubbard et al. (2017) compared soil community structure between wild-type and clock mutant *A. thaliana* plants and observed different community structures between day and night time points as well as an altered microbial community in clock mutant soil. Further to this, the beneficial effects of root-colonising bacteria are influenced by photoperiod. Kloepper et al. (2007) grew pepper (*Capsicum annuum*) plants under long (16 h) and short (8 h) light cycles and found that the beneficial *Bacillus subtilis and Bacillus amyloliquefaciens* on the roots were only able to elicit

significant increases in plant growth under long day conditions. This suggests that photoperiod regulates bacterial induced growth-promotion, however, it does not provide much insight into whether this is due to the photoperiod directly affecting plants or bacteria.

Reinforcing the potential for bacterial circadian clocks, is their known ability to detect changes in environmental stimuli such as light and temperature. Bacteriophytochromes are bacterial phytochromes that are modulated by red and far red light and are found in a range of bacteria including non-photosynthetic species such as *Pseudomonas aeruginosa, Agrobacterium tumefaciens* and *Deinococcus radiodurans* (Davis et al. 1999; Bhoo et al. 2001). Of more interest is the fact that many of the species in the *Rhizobiaceae* bacterial family, which can form plant symbionts, have bacteriophytochromes (Rottwinkel et al. 2010). This suggests a benefit of light sensing in the soil environment and may link to the benefit of entraining to the diurnal variations in plant photosynthetic activity. Recently, evidence for functional blue light photoreceptors in non-phototrophic bacteria has started to accumulate. Starting with the discovery of the light, oxygen, voltage (LOV) domain in *B. subtilis,* the first documentation outside of plants (Losi et al. 2002). Since then, a repertoire of other blue light sensing photoreceptors have been found in a variety of bacterial families including the photoactive yellow protein (PYP) and FAD (BLUF) domain proteins (Gomelsky & Klug 2002; Tschowri et al. 2009; Memmi et al. 2014). Many of these blue light sensing bacteria form associations with plant roots in the soil. Examples include the beneficial rhizobacterium *Burkholderia phytofirmans* which possesses genes with a PYP domain and *B. subtilis* with a LOV domain in the YtvA protein (Kumauchi et al. 2008). The pea-nodulating endosymbiont *Rhizobium leguminosarum* uses LOV domain light receptors to regulate exopolysaccharide production and amount of root nodulation, however, it is also hypothesised that light sensing could allow rhizobia to sense the time of day and position within the environment to optimise root infection (Bonomi et al. 2012).

1.2. An economically important species: *Bacillus subtilis*

Microbial production of secondary metabolites is economically important for industry and agriculture, yet how their circadian biology affects this process remains undetermined. *Bacillus* is the most abundant genus of gram-positive soil bacteria, with up to 95% of soil sequences originating from *Bacillus* species (Garbeva et al. 2003). The following section aims to review the ecology and economic importance of *Bacillus subtilis* for agriculture, pharmaceutical and other industries.

1.2.1. Ecology and genomics

Bacillus subtilis is a rod-shaped, non-pathogenic Gram-positive bacterium that has become a model organism for microbial studies for over a century. It can be isolated from a myriad of environments: from Antarctic and forest soils to the gastrointestinal tracts of humans and ruminants (Holding et al. 1965; Heal et al. 1967; Siala et al. 1974; Macfarlane et al. 1986; Hong et al. 2009). One reason accredited to its widespread distribution is the ability to produce highly resistant endospores as a survival mechanism against environmental stresses. These spores provide a strategy for the species to cope with, and spread from, unfavourable local conditions to new surroundings (Nicholson et al. 2000). Their extreme environmental resistance has even led to their consideration as possible candidates for transfer of life to other planets (Lindsay & Murrell 1983; Horneck 1993).

B. subtilis was once thought to only be a strict aerobe, until Priest (1993) published a review which prompted several investigations into anaerobic respiration in the bacterium, demonstrating that *B. subtilis* can carry out anaerobic respiration using nitrate as a terminal electron acceptor (Cruz Ramos et al. 1995; Glaser et al. 1995; Nakano et al. 1996; Cruz Ramos et al. 2000; Reents et al. 2006). Genomics has also paved the way for significant steps to be made in *Bacillus* research: genome sequencing by Kunst et al. (1997) was extremely valuable in that it identified several components involved in this respiration, many were homologues of the components of the *E. coli* anaerobic system. One example was the FNR (fumarate and nitrate reductase) transcriptional activator which is responsible for the switch from aerobic to anaerobic respiration. This ability to switch respiratory mechanisms also partially explains *B. subtilis'* success in a range of environments, particularly in the anaerobic conditions of animal digestive systems.

Interestingly, sequencing of the *B. subtilis* genome revealed a large proportion of genes that encode pathways for the utilisation of plant-derived molecules such as opines, providing evidence of the close association of the bacteria with roots (Kunst et al. 1997). Subsequent studies have successfully isolated it from the roots of multiple crop species, including beets (*Beta vulgaris*), carrots (*Daucus carota*), radish (*Raphanus sativa*), canola (*Brassica napus*) wheat (*Triticum aestivum*) and tea bushes (*Camellia sinensis*), confirming this close association with plant roots in the rhizosphere (Pandey & Palni 1997; Germida et al. 1998; Fall et al. 2004). These associations were often still found even in unfavourable temperature conditions.

1.2.2. Pharmaceutical and industrial applications

Microbial production of secondary metabolites by *B. subtilis* has led to its use for the synthesis of a wide range of important agricultural, pharmaceutical and other industrial products. The bacterium

dedicates approximately 4% of its genome to the production of secondary metabolites (Kunst et al. 1997). A number of these products are potent fungal and bacterial inhibitors, in fact, the potential for *B. subtilis* to produce peptide antibiotics has been known for over 50 years (Katz & Demain 1977). The species produces over two dozen antibiotics, including lantibiotics (peptide antibiotics), such as subtilin, and lipopeptides in the surfactin, iturin and fengycin families. Some of these are produced by a variety of strains such as surfactin, whereas others have strain-specific production (Stein 2005).

The lantibiotic subtilin shows strong antimicrobial activity against Gram-positive bacteria (Salle & Jann 1945; Hassall 1948; Moore & Wooldridge 1950). Subtilin acts by inducing pores in the cell membrane and cytoplasmic membrane vesicles in other bacterial cells, resulting in an efflux of amino acids and ultimately cell death (Schüller et al. 1989; Bierbaum & Sahl 2009). Perhaps of more commercial interest is the lipopeptide antibiotic surfactin (Bernheimer & Avigad 1970). In addition to its antimicrobial properties; antiviral, antitumor, anticoagulant and fibrinolytic effects have been observed (Kameda et al. 1974; Vollenbroich et al. 1997; Kracht et al. 1999; Kim et al. 2006; Kim et al. 2007). However, commercial use has been limited by the high cost of production and low yields (Shaligram & Singhal 2010)

Bacillus has been used worldwide in probiotic treatments, owing to its antibiotic effects. It has antagonistic properties against *H. pylori* and *Campylobacter* spp.*,* which cause several human gastrointestinal diseases including stomach cancer (Sorokulova et al. 1997; Pinchuk et al. 2001). The use of *Bacillus* species in aquaculture is also expanding, particularly in areas with intensive fish farming, due to its effectiveness at inhibiting pathogenic bacteria and enhancing growth in multiple shrimp and fish species (Vaseeharan & Ramasamy 2003; Zokaeifar et al. 2012; Del'Duca et al. 2013; Hai 2015).

Bacillus species also hold commercial interest due to their natural ability to act as protein secretion factories, producing enzymes of biotechnological importance, such as α- amylase, proinsulin and lipase (Palva 1982; Lesuisse et al. 1993; Olmos-Soto & Contreras-Flores 2003). Amylase from *B. subtilis* can be used in baking, for starch hydrolysis in the beverage industry, as a bio-detergent for carbohydrate stains, for desizing in the textile industry and more (Schallmey et al. 2004; Singh et al. 2016). Additionally, *B. subtilis* produces milk clotting enzyme (MCE) and can be optimised for use in cheese production (Wu et al. 2013). Further enhancements to extracellular enzyme production by *Bacillus* species are only set to increase their use in industry.

1.2.3. Agricultural applications 1: *B. subtilis* as a plant growth-promoting rhizobacterium

As mentioned, *B. subtilis* forms associations with plant roots and has benefits for both maintaining the health and growth of field crops. In fact, *B. subtilis* is classified as a plant growth-promoting rhizobacteria (PGPR), a reflection of its numerous beneficial traits including enhancing plant growth and suppression of pathogens. The latter can be through secretion of antimicrobial compounds, competition for resources and activation of plant host defences.

The nature of the interaction between plants and *Bacillus* spp. is complex and involves signalling between the host and the bacteria. The bacteria may first be attracted towards the roots through the secretion of root exudates such as L-malic acid (Rudrappa et al. 2008). Subsequently, for successful colonisation the bacteria form adhesive biofilms on the root surface. Recognition of plant polysaccharides in the cell wall activates matrix genes which results in the production of extracellular polymers to facilitate attraction and form the matrix between cells (Beauregard et al. 2013) (Figure 1.3.). After colonisation, the PGPR can utilise plant root exudates whilst benefitting the plant through growth enhancement and disease suppression.

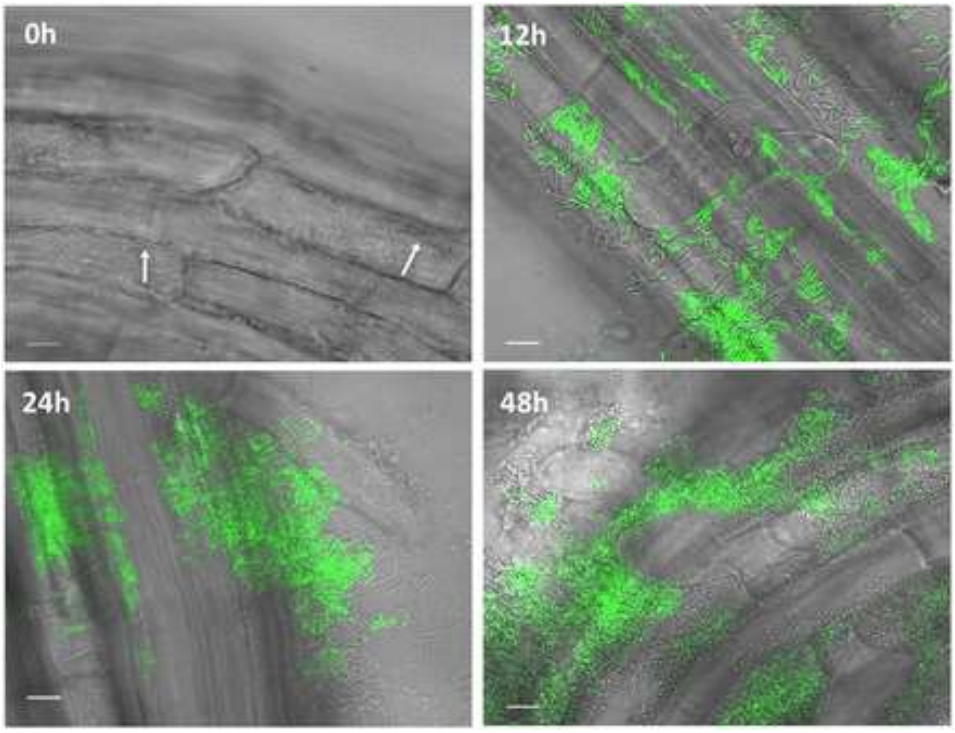

Figure 1.3. *B. subtilis* cells colonising *A. thaliana* roots. Images taken at different time points post-inoculation. Green fluorescence is the result of bacterial cells expressing YFP under the control of the matrix gene *tapA*. (Reproduced from Beauregard et al. 2013).

Numerous studies have found evidence for increased plant growth with different *Bacillus* species, including *B. subtilis*. For example, Swain and Ray (2006) found that root inoculation of chickpea (*Cicer arietinum*) with *B. subtilis* strains CM1 and CM3 resulted in root elongation of chickpea seedlings up to 70-74% compared to untreated controls. This may have been due to the secretion of phytohormones from the bacteria, since some *Bacillus* species have been previously reported to produce indole acetic acid (IAA), cytokinins, and gibberellins with strong growth-promoting activity (Idris et al. 2004; Arkhipova et al. 2005; Gutiérrez-Mañero et al. 2008). However, whether this increase in root length led to an increase in chickpea yield, was not studied. The *B. subtilis* PTS-394 strain was also shown to increase root length and plant height in tomato, this time by up to 18.4% and 18.3% respectively. Once again, the subsequent effects on actual crop yield were not measured and the study also found that a high amount of colonisation by the bacteria (exceeding 7 x10^7 CFU/g of fresh root) slightly inhibited root growth, suggesting that high bacterial densities may negatively affect the plant in respect to growth (Qiao et al. 2017). Studies in other crops have investigated the effects on yield. For example, when seeds of saffron (*Crocus sativus*) were inoculated with *B. subtilis* FZB24, leaf length, flowers per corm and crucially, stigma biomass, all significantly increased compared to untreated controls. This increase in stigma biomass represented a 12% increase in yield (Sharaf-Edin et al. 2008). Experiments by Xie et al. (2014) gave more insight in to some compounds which may be the causative agents of these growth increases. Application of *B. subtilis* strain OKB105 increased the length of tobacco roots by 55.9% compared to controls and mutant library and HPLC analysis was used to show that increased levels of spermidine, a common polyamine produced by plants, was the chemical responsible for increased plant growth.

1.2.4. Agricultural applications 2: anti-pathogenic effects of *B. subtilis* on plants

Alongside the direct impacts on growth, *Bacillus* can also enhance growth indirectly by suppressing fungal and bacterial diseases in plants. Yánez- Mendizábal et al. (2012) reported on *Bacillus* antifungal activity against peach brown rot (*Monilinia* spp.), a pathogen causing significant postharvest losses in stone crop fruits (Hong et al. 1997). The antifungal activity was shown to be due to fengycin-type lipopeptides, which were produced by the *B. subtilis* CPA-8 strain. Fengycin lipopeptides are also produced by the M4 strain and offer protection against grey mould (*Botrytis cinerea*) of apple post-harvest and against damping-off caused by *Pythium ultimum* in bean seedlings (Ongena et al. 2005a). *Botrytis* and *Pythium* are both major agricultural fungal diseases: *Botrytis* infects over 200 plant species and causes losses of $10-100 billion worldwide (Boddy 2016). *Pythium* also has a wide host range of over 150 species, and is extremely environmentally resistant, having been shown to survive in air dried soil for 12 years (Hendrix & Campbell 1973).

Another agricultural pathogen is the bacteria *Pseudomonas syringae,* with over 40 pathovars and 80 host species, including major crops such as tomato (Hirano & Upper 2000). Bais et al. (2004) showed that *B. subtilis* formation was able to protect *Arabidopsis thaliana* against *P. syringae* through secretion of the antimicrobial lipopeptide, surfactin. Recombinant strains have also been generated which show enhanced antimicrobial effects. For example, *B. subtilis* strain BBG100 was modified so that the promoter of the mycosubtilin operon was substituted with a constitutive promoter from *Staphylococcus aureus,* resulting in a 15-fold higher mycosubtilin production rate than the parental strain. When tested for its biocontrol potential, both the wild-type and over-expressor supernatents were able to induce growth inhibition in three phytopathogenic fungi: *B. cinerea, Fusarium oxysporum* and *Pythium aphanidernatum.* However, the over-expressor was significantly more effective against the three pathogens. When experiments were conducted in whole tomato seedlings, the wild-type strain failed to protect the seedlings from *Pythium* infection, whereas pre-treatment with BBG100 significantly increased seedling emergence (Leclère et al. 2005). It is difficult to distinguish whether this reduction in disease is due directly to antibiotic production by the bacteria or if it is the indirect result of inoculation causing induced systemic resistance (ISR) in the plants.

1.2.5. Defence signalling between *B. subtilis* and plant hosts

Induced systemic resistance (ISR) is the induction of defences triggered throughout the plant and results in changes such as production of antimicrobial phytoalexins, thickening of the cell wall, accelerated stomatal closure and increased expression of genes involved in other defence pathways. These responses have been demonstrated in over 15 plant species with several different rhizobacteria including *Psuedomonas, Bacillus and Trichoderma* (Wang et al. 2005; Schuhegger et al. 2006; Van Loon & Bakker 2006). Signals from the bacteria activate the process which is then commonly mediated by the phytohormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) via the NPR1 plant receptor (Kloepper et al. 2004; Verhagen et al. 2004; Wu et al. 2012).

Numerous studies have investigated this defence signalling between the bacteria and plant. For example, Rudrappa et al. (2008) showed that infection by *P. syringae* in *Arabidopsis* leaves induced root secretion of malic acid which in turn resulted in a 4-fold increase in *B. subtilis* FB17 colonisation in the roots, to assist in protecting against the plant pathogen. Later, Fousia et al. (2015) investigated the effect of *B. subtilis* QST 713 from the commercial biofungicide Serenade Max (Bayer CropScience) and found spraying bacteria onto tomato seedlings conferred protection against *P. syringae* and increased plant height compared to control plants. Strain QST 713 produces the strong antibiotic surfactin which is likely to be one mechanism involved in this plant defence. The study also

found inoculation enhanced expression of plant genes involved in defence pathways, such as for salicyclic acid and ethylene/jasmonic acid synthesis, which builds on the evidence for signalling between the bacteria and plant, and ISR. Similar results were found in a study using the BSCBE4 strain to control damping off by *Pythium aphanidermatum* in hot pepper. In this case, the strain increased levels of plant defence-related enzymes such as peroxidase and polyphenol oxidase by 3-4 fold, inducing systemic resistance of the host to the pathogen (Nakkeeran et al. 2006). In addition, Ongena et al. (2005b) used cDNA-AFLP to find that following root colonisation in tomato and cucumber by *B. subtilis* strain M4, 6.2% and 3.7 % of all genes were upregulated and 4.7% and 6.2% were downregulated, respectively. These changes in gene expression were associated with reduced disease incidence caused by the fungal pathogens *Colletotrichum lagenarium* (on cucumber) and *Pythium aphanidermatum* (on tomato). Interestingly, in the aforementioned paper by Xie et al. (2014), although root colonisation by *B. subtilis* OKB105 increased root length in tobacco, ethylene content in the plant was significantly lower than the control. PGPRs have been shown to synthesise the bacterial enzyme 1-aminocyclocpropane-1-carboxylate (ACC) deaminase which reduces the amount of ethylene in the plant by hydrolysing the ethylene precursor ACC (Glick et al. 1998). This is beneficial for the plant under stress conditions since it can alleviate the negative impacts of stress-induced ethylene production, such as growth inhibition, premature ripening, and senescence, all of which can potentially reduce plant productivity (Burg 1973). The ability to produce ACC deaminase has been shown in several *Bacillus* species, including *B. subtilis* (Kumar et al. 2012a; Xu et al. 2014; Khan et al. 2016).

In the face of changing climates, it is also important to consider the effects of PGPR under different stress conditions. Mohamed and Gomaa (2012) studied the effects of *B. subtilis* on the growth and pigment composition of radish plants (*Raphanus sativus*) under salinity stress. Root and leaf biomass significantly increased in inoculated plants under both unstressed and stressed conditions. The increase in growth was attributed to the reduction in stress-produced ethylene, although this was not tested directly. Interestingly, treatment with *B. subtilis* counteracted some of the negative effects of salt stress on nutrient content in the plants. Ordinarily, one effect of salinity is decreased protein content of the plant due to the removal of potassium ions, which are essential for protein synthesis (Mohamed & Gomaa 2012). However, inoculation increased protein levels. It should be noted that these results were for co-inoculation with *Psuedomonas fluorescens*, so *B. subtilis* alone may not produce consistent results.

Clearly there is evidence that the bacteria can signal to the plant to activate defence pathways, but this interaction appears to be reciprocal, with the plant able to activate defence genes in root bacteria. For example, Kobayashi (2015) demonstrated that *B. subtilis* grown with an uprooted weed

on solid media forms mucoid colonies surrounding the plant roots. This growth occurs in response to the release of methyl salicylate from the roots, a chemical emitted as a defence signal in response to infection. Consequently, methyl salicylate from the plant induced biosynthesis of the antibiotics bacilysin and fengycin from *B. subtilis* which inhibited the attacking fungus *Fusarium oxysporum.*

Together, these studies demonstrate that *B. subtilis* provides a viable alternative to chemical pesticides and herbicides for reducing plant diseases and enhancing plant growth. Several *B. subtilis* biocontrol agents are already on the market for anti-fungal and anti-bacterial protection. Some examples include the *B. subtilis* strain QST 713 in Serenade ASO (Bayer CropScience) which targets the causative agent of grey mould (*Botrytis cinerea*), Companion® (Growth Products) and Kodiak® (Gustafson Inc), which use the GB03 strain to prevent and control a broad spectrum of root and foliar pathogens such as *Phytophthora, Pythium, Fusarium* and *Rhizoctonia* (Brannen & Kenney 1997; McSpadden et al. 2002). Both biocontrol agents are effective for use in a range of crops making them excellent for commercial application.

1.3. Project aims

With the global population predicted to increase to 9.1 billion by 2050, a rise that is set to require a 70% increase in food production, it is more important than ever to understand mechanisms by which crop yields can be improved (FAO 2009). As discussed in this chapter, it is evident that rhizobacteria such as *B. subtilis* hold strong potential for enhancing plant growth and suppressing disease in agricultural systems. Nevertheless, whether these organisms possess a circadian clock and whether their root colonisation influences plant circadian biology remains undetermined. Further work is required to uncover the interplay of this relationship which may reveal bacterial-induced changes to host plant rhythms that in turn could alter plant fitness and subsequently yield.

This work investigates circadian rhythms in *B. subtilis* and explores evidence for signalling of circadian timing information between bacteria and *Arabidopsis thaliana* host plants. These aims are approached through targeting the following objectives:

1. Investigation into the influence of plant circadian rhythms on *B. subtilis ytvA* oscillations.
2. Impacts of root colonisation by *B. subtilis* on circadian rhythms in *A. thaliana* shoots.

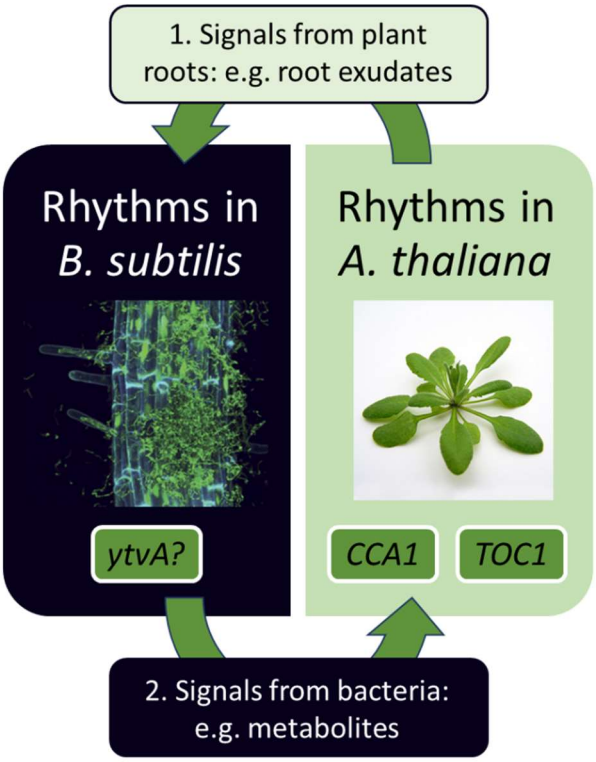

Figure 1.4. Model for the hypothesised signalling of circadian timing information between *B. subtilis* and *A. thaliana* host plants. White bordered rectangles represent known circadian clock genes in plants (*CCA1* and *TOC1*) and potential genes in the bacterial circadian system (*ytvA*). Labelled arrows demonstrate entrainment stimuli which may influence circadian rhythms in the plant host or symbiotic bacteria.

As illustrated in Figure 1.4., rhythms in *B. subtilis* were tested by monitoring expression of the potential bacterial clock gene *ytvA.* This builds on the work by Prof Martha Merrow's group at the University of Munich, who have obtained unpublished evidence that *ytvA* gene expression oscillates when entrained to light and temperature cycles. Conversely, changes to *Arabidopsis* rhythms following bacterial root inoculations were monitored via measuring gene expression of core clock genes such as *CCA1* and *TOC1.*

Chapter 2: Materials and Methods

Chemicals were purchased from Thermo Fisher Scientific, Sigma-Aldrich, Melford and VWR. Media was made up in distilled water and autoclaved at 121° C for 22 min.

2.1. Plant materials and growth conditions

2.1.1. *Arabidopsis* seed stocks and sterilisation

Wild-type *Arabidopsis thaliana* seeds were obtained from Nottingham Arabidopsis Stock Centre (stock N600000, Col-0 background). Other lines used were *CCA1::LUCIFERASE* (Col-0 background, Noordally et al. 2013) and *TOC1::LUCIFERASE* (Col-4 background, gift of Anthony Hall).

Seeds were surface sterilised using a method adapted from Belbin et al. (2017). Seeds were first exposed to 70% ethanol (v/v) for 1 min, followed by 20% sodium hypochlorite (v/v) for 10 min, and subsequently washed twice with sterile distilled water (sdH_2O). They were then pelleted by centrifuging for 15 s at 5000 rpm, resuspended in 0.1% agar (w/v) and transferred individually onto half-strength Murashige and Skoog nutrient mix (basal salts without vitamins, Duchefa Biochimie, the Netherlands) in 0.8% (w/v) agar. 7 seeds were sown per 60 mm diameter petri dish, according to Figure 2.1.

CCA1::LUC and *TOC1::LUC* reporter plants for use in experiments to test effects of media on circadian rhythms were grown in sterile plastic rings embedded in the MS medium (Figure 2.2.) (Dodd et al. 2014). 10-15 seeds were pipetted per ring. All seed plates were sealed with micropore tape and left to stratify in darkness at 4° C for 3 days.

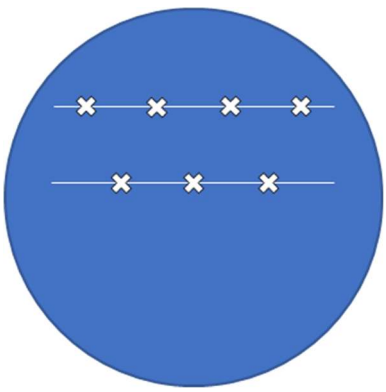

Figure 2.1. Template used for positioning seeds. "X" symbols represent where seeds were plated onto the 60 mm plate.

Figure 2.2. Circadian clock luciferase reporter seedlings cultivated in sterile plastic rings embedded in MS in 90 mm plates. 10-15 seeds were sown per well.

2.1.2. Plant growth and entrainment conditions

Petri dishes harbouring seeds were transferred to Panasonic MLR-352 growth chambers (Osaka, Japan) and positioned vertically to ensure roots grew across the media surface (Figure 2.3.). Plants growing in plastic rings were positioned horizontally as imaging of roots was not required.

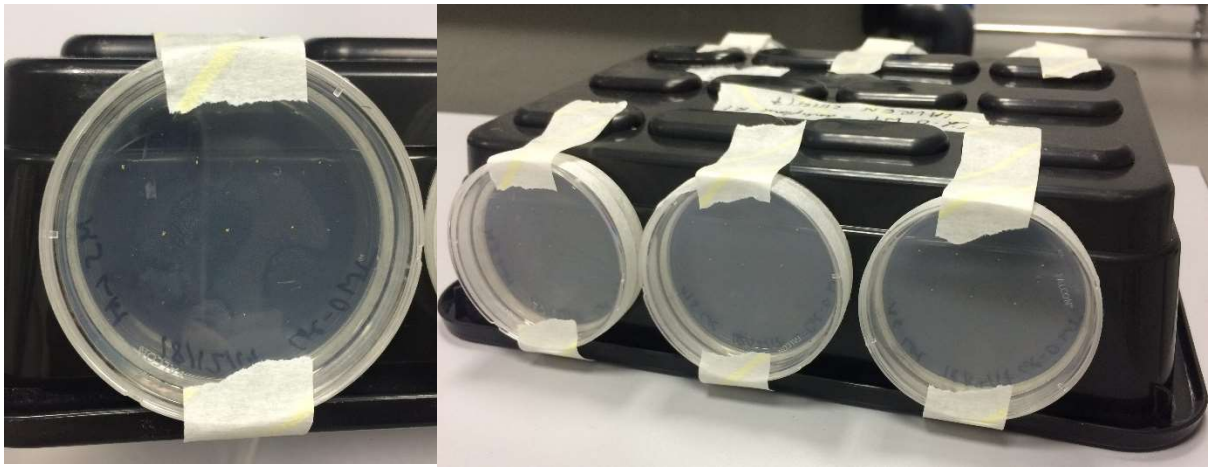

Figure 2.3. Vertical positioning of seed plates for plant growth. For experiments involving root inoculation and imaging, plants were growth vertically to ensure root growth over media surface.

Two different sets of lighting conditions were used for entraining plants. These were termed phase entrainment or antiphase entrainment. Phase entrained plants were under 12 h:12 h light:dark (LD) cycles under 80 $\mu molm^{-2}s^{-1}$ white light from 9am-9pm. Antiphase entrained plants were given the opposite entrainment regime, with the lights on 9pm-9am (DL). This enabled plants with antiphase rhythms to be generated. Both treatments were maintained at 19 °C constant temperature.

2.2. Culture of *B. subtilis*

2.2.1. Bacterial strains

All strains used in this study are listed in Table 2.1. These were kindly donated by the Merrow lab at the Ludwig Maximilian University of Munich and were constructed by Ruud Detert Oude Weme, Ákos Kovács and Oscar P. Kuipers at the University of Groningen, the Netherlands.

Transgenic reporter strains were derived from the *B. subtilis* 168 strain, a domesticated strain which has retained some biofilm-forming and plant attachment abilities (Gallegos-Monterrosa et al. 2016). Each of these 7 strains have the bioluminescent *luxABCDE* reporter under the control of one of the following bacterial gene promoters: *resE, ykoW, ydfH, yycG, phoR, kinC* or *ytvA*. The *luxABCDE* reporter generates an endogenous bioluminescence signal, thus enabling light output to be monitored as a measure of the bacterial promoter activity.

Strain	Genotype
168 (Domesticated laboratory strain)	Δ*trpC*
P_{resE}-*luxAB*	Δ*trpC; amyE::* P_{resE}-*luxABCDE* Cm^R
P_{ykoW}-*luxAB*	Δ*trpC; amyE::*P_{ykoW}-*luxABCDE* Cm^R
P_{ydfH}-*luxAB*	Δ*trpC; amyE::*P_{ydfH}-*luxABCDE* Cm^R
P_{yycG}-*luxAB*	Δ*trpC; amyE::*P_{yycG}-*luxABCDE* Cm^R
P_{phoR}-*luxAB*	Δ*trpC; amyE::*P_{phoR}-*luxABCDE* Cm^R
P_{kinC}-*luxAB*	Δ*trpC; amyE::*P_{kinC}-*luxABCDE* Cm^R
P_{ytvA}-*luxAB*	Δ*trpC; amyE::*P_{ytvA}-*luxABCDE* Cm^R

Table 2.1. Strains used in this study. Cm: chloramphenicol.

2.2.2. Bacterial growth media and conditions

For routine growth, each strain was streaked from frozen glycerol stocks onto LB agar (50 g Miller's LB broth with agar/L) plates and incubated at 37 °C overnight before storage at 4 °C. Miller's LB has a higher salt content than other types of LB media (10 g/L NaCl compared to 5 g/L for Lennox LB and 0.5 g/L for Luria LB). To generate liquid cultures for growth curve experiments, an inoculation loop was used to transfer a mix of colonies from LB plates to 20 ml liquid LB (10 g tryptone, 5 g yeast, 10 g NaCl, 1 ml 1M NaOH /L). These cultures were maintained at 37 °C in an orbital shaker at 110 rpm under ambient lighting conditions. OD_{600} values were measured every 30 min using a spectrophotometer (Biochrom Biowave II; Biochrom Ltd, UK) until OD_{600} absorbancy reached the maximum reading of > 2.5. For transformed strains, all LB media was supplemented with 25 µg/ml chloramphenicol (Cm).

For circadian entrainment of bacteria, 10 ml liquid cultures were grown on either static or shaking (depending on the experiment) within a plant growth cabinet (Microclima Economic Lux; Snijders Labs, the Netherlands) for 3 days. A 12 h:12 h LD or DL cycle of white light was used to entrain bacteria to phase or antiphase cycles respectively, and the temperature was maintained at 29 °C (Zheng Chen 2018, pers.comm, 8 February). Bacterial culture without entrainment involved wrapping the liquid cultures within a 6-well plate with foil to prevent light entrainment. The static plate was then incubated in the 29 °C cabinet with the entrained bacteria or in an incubator at 37 °C for earlier experiments with non-entrained bacteria.

2.2.3. Culture and staining of static biofilms

Biofilms of each strain were cultured on microscope slides as follows, using a method adapted from Merritt et al. (2005). All 7 transgenic strains were grown in liquid cultures to stationary phase (OD_{600} > 2.0) as described above. Next, a 20 µl aliquot of this culture was diluted in 20ml LB (+ Cm 25 µg/ml) in a falcon tube. A sterilised glass microscope slide was inserted, and the culture was placed at an angle in a tube rack held by a cardboard support and incubated at 37 °C for 48 h (Figure 2.4.). The biofilm formed at the air-liquid interface and adhered to the microscope slide allowing for staining and imaging of biofilms later.

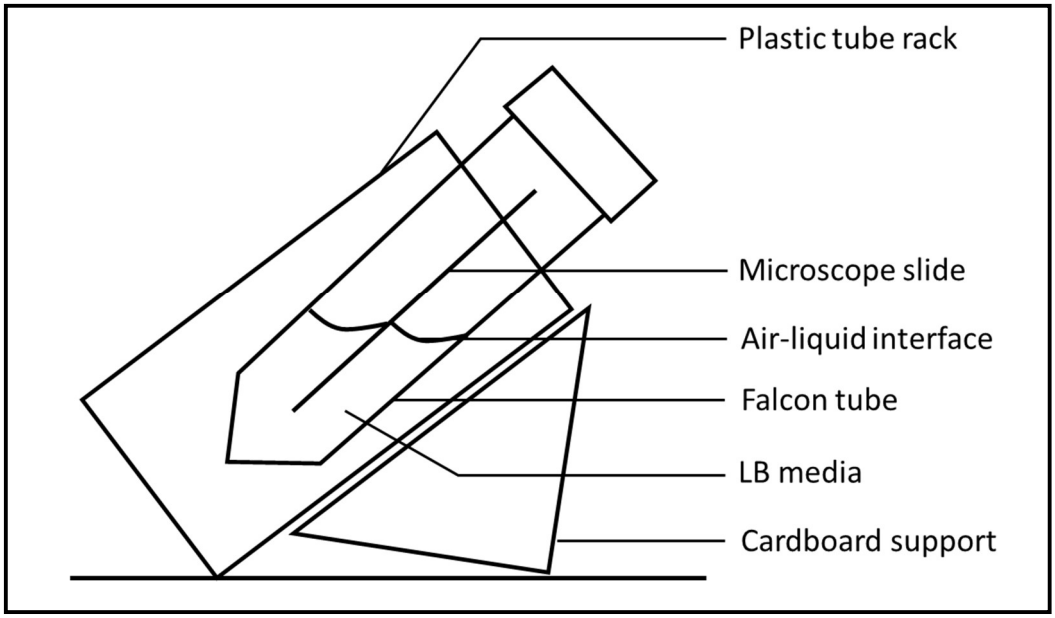

Figure 2.4. Schematic for microscope slide assay of biofilm formation in *B. subtilis* (side view)**.** Transgenic strains were grown in LB in an angled falcon tube. Biofilm formation occurred at the air-liquid interface and adhered to the inserted microscope slide.

2.3. Bioluminescence timecourse imaging

Several different methods and time course programs were used for bioluminescence imaging experiments. These can broadly be divided into 2 approaches corresponding to the 2 project objectives.

2.3.1. Imaging of bioluminescent transformed bacteria

Transformed bacterial strains did not require treatment with luciferin prior to imaging as the *luxABCDE* reporter generates a bioluminescence signal in absence of an exogenous luciferin substrate.

Image capture was again conducted using a Photek ICCD218 high resolution intensified CCD photon counting camera controlled by IMAGE32 software (Photek, East Sussex, UK). The virtual neutral density filter that was used varied according to the bacterial strain to ensure photon counts were maintained below 1500 counts/s. For experiments measuring light output from the *ytvA* strain on roots the program was as followed: 10% filter, 10 min integration every 30 min for 7 days. For experiments involving root inoculation prior to imaging, see method development in 3.2.

2.3.2. Luciferin treatment and imaging of plants with bioluminescent reporters of the circadian clock

The evening before imaging, approximately 13-day old seedlings were each dosed across the shoot and roots with 100 µl of 5 mM or 10 mM luciferin (potassium salt of D-luciferin; Melford Laboratories Ltd, Ipswich, UK) depending on the experiment. Reporter plants grown in sterile plastic rings for testing of the effect of bacterial grown media upon the plant circadian oscillator were instead treated with 50 µl of 5 mM luciferin per well. This was followed by 50 µl of media 15 min later.

The next morning, plants were again imaged using a timecourse of imaging collected with the ICCD photon counting camera. Images were captured every 30 min, with an integration time of 5 min using either a 1% or 10% virtual neutral density filter depending on the experiment. Between image capture, plants were under constant equal red-blue light totalling 50 $\mu molm^{-2}s^{-1}$ (LL). During data extraction, the first 2 min of signal within each integration was removed to eliminate chlorophyll autofluorescence (Gould et al. 2009; Dodd et al. 2014).

2.4. Statistical analysis

BioDare2 (biodare2.ed.ac.uk) was used to calculate circadian phase and period estimates from photon counting data (Zielinski et al. 2014). Fast Fourier transform (non-linear least square method; FFT NLLS) analysis and Maximum Entropy Spectral Analysis (MESA) were applied to time-course series to generate period estimates and calculate the associated relative amplitude error (RAE). RAE values give an indication of rhythm robustness, where values closest to 0 are robust and close to 1 are weak (or no) rhythmicity (Plautz et al. 1997; Zielinski et al. 2014). The first 24 h of each timecourse was omitted as during this time there may be transition effects due to the transfer of plants from LD cycles to LL.

To test for statistically significant differences in phase and period between treatments, a one-way analysis of variance (ANOVA) was conducted using IBM SPSS® software. A post-hoc Tukey test was also used to make multiple pairwise comparisons.

2.5. Microscopy

2.5.1. Light microscopy

Crystal violet (CV) is a common stain for gram-positive bacteria such as *B. subtilis*, I tested whether it might be used as a potential indicator of biofilm formation in the 7 strains (Bartholomew & Finkelstein 1958; Fall et al. 2004; Ma et al. 2017). Following each of the staining methods detailed below, samples were imaged using a Leica ICC50 HD light microscope and images were captured using the Leica Application Suite V4 software (Leica Microsystems, UK).

2.5.1.1. Crystal violet staining of biofilm on slides

The simple method of crystal violet staining adapted from Merritt et al. (2005) was selected for staining the biofilms that had formed on microscope slides. The slides were carefully removed from the culture so that the biofilm that had formed was not dislodged. Samples were heat fixed by passing through a Bunsen burner twice and submerged in 0.1% CV solution (w/v) for 10 min. The 0.1% CV solution was made by mixing a 10% stock solution (diluted in ethanol) with a 0.1% solution of ammonium oxalate. Samples were then washed by dipping in 2 successive water baths, left to air dry and any remaining water was blotted with tissue.

2.5.1.2. Inoculating and staining *Arabidopsis* roots for crystal violet staining

Three 20-day old Col-0 plants were transferred to each well in a 6-well plate, containing 5 ml solid MS agar. The roots in each well were then inoculated with different bacterial solutions by streaking with an inoculation loop. Inocula were produced by culturing the *ytvA* strain in LB until $OD_{600} > 2$, centrifuging an aliquot of the culture for 5 min at 1500 rpm to pellet the bacteria before resuspension and dilution (1:100) in either liquid half-strength MS or half-strength MS + 1% tryptone (w/v). The inoculated plants were then incubated horizontally (in the plant growth conditions previously described) for 4 days to allow enough time for biofilm formation on the roots (Bais et al. 2004; Beauregard et al. 2013; Chen et al. 2013).

One plant from each inoculation treatment was transferred to a sterilised microscope slide, and the shoot and excess root were removed. Following this, the roots were given one of two CV staining methods (Table 2.2.). The simple CV staining method (used for samples 1, 2, 3 and 5) involved pipetting 0.1% CV (w/v) onto roots to cover and incubating for 10 min. This was followed by rinsing with sdH_2O until the liquid ran clear and then blotting dry. For samples 4 and 6 the traditional method of Gram staining was used (Gram 1884). Once again this involved a 10 min CV stain, followed by rinsing with sdH_2O. Then the root was covered with Gram's iodine solution (6.66 g

potassium iodide, 3.33 g iodine, 10 g sodium bicarbonate/L) for 30 s, rinsed again, covered with decolouriser (1:1 acetone:ethanol) for 5 s, rinsed and blotted dry.

Sample number	Root inoculation treatment	Staining treatment
1	Control: MS	10 min CV
2	Control: MST	10 min CV
3	*ytvA* + MS	10 min CV
4	*ytvA* + MS	10 min CV + Gram's iodine + decolouriser
5	*ytvA* + MST	10 min CV
6	*ytvA* + MST	10 min CV + Gram's iodine + decolouriser

Table 2.2. Treatments used for the inoculation of *Arabidopsis* roots with *B. subtilis* and subsequent crystal violet (CV) staining. Roots were streaked with *ytvA::luxABCDE* strain grown to OD_{600} > 2.0, resuspended 1:100 in half-strength MS liquid (MS) or half-strength MS liquid + 1% tryptone (w/v) (MST). Staining treatments were either basic staining with 0.1% CV for 10 min or traditional Gram's staining method with subsequent washing with Gram's iodine solution and decolouriser.

2.5.2. Confocal microscopy

The green-fluorescent nucleic acid stain SYTO™13 (Invitrogen, USA) was used to stain biofilms that had formed on plant roots as used in similar studies (Rudrappa et al. 2008).

5 mM SYTO™13 stock solution was diluted to 15 µM in sdH_2O. Inoculated roots were covered with 500 µl dye and incubated for 15 min followed by gently rinsing the sample in sdH_2O. It should be noted that several combinations of dye concentrations (0.5, 5, 15 µM) and incubation times (5, 15, 30 min) were tested with 15 min staining and a 15 µM solution being optimal. Plants were transferred to sterile microscope slides, excess root and shoot was removed, the root was mounted in sdH_2O and covered with a 0.17 mm glass coverslip bridge. Samples were wrapped in foil before imaging to prevent light degradation of the fluorescent dye. Presence of *B. subtilis* biofilm on the plant roots was determined by imaging with a Leica TCS SP5 confocal microscope fitted with an Argon laser and under the control of Leica LASAF software (Leica Microsystems, UK). Root samples were excited with the laser at 488 nm wavelength and emissions were collected at 500-550 nm.

Chapter 3: Influence of plant circadian rhythms on *B. subtilis ytvA::luxABCDE* oscillations.

3.1. Introduction and aims

To date, there is very little evidence for circadian rhythms in non-photosynthetic bacteria, with few reports of periodicity in growth (Thompson et al. 1995; Soriano et al. 2010; Paulose et al. 2016). Only the phylogenetic studies by Dvornyk et al. (2003) and Loza-Correa et al. (2010) have reported on circadian *kaiB* and *kaiC* gene homologues in other bacterial groups such as Proteobacteria, Thermotogae and Chloroflexi. Normal functioning of the cyanobacterial clock is altered in the absence of *kaiA* so it is unknown if the hypothetical non-cyanobacterial clock functions without *kaiA* or uses a different system (Nishimura et al. 2002). Since non-photosynthetic bacteria are of significant economic importance, providing several industrially-relevant chemicals and pharmaceuticals and serving as anti-pathogenic and growth-enhancing agents in agriculture, it is important to understand their circadian biology (Schallmey et al. 2004; Stein 2005; Swain & Ray 2009).

Free-living photosynthetic bacteria benefit from possessing circadian oscillators to accurately time physiological and behavioural responses to predictable daily changes in environmental variables (Ouyang et al. 1998; Woelfle et al. 2004). Additionally, I speculate that circadian oscillators in non-photosynthetic bacteria in association with plants might confer a fitness advantage. Circadian clocks may allow bacteria to synchronise responses to rhythmic cues from the host plant, such as timing the synthesis of components required for metabolising plant exudates in the bacteria with rhythmic secretion of exudates from plant roots. Understanding circadian rhythms in non-photosynthetic bacteria such as *B. subtilis* could provide new insights into how these bacteria interact with host plants and with other microorganisms in the rhizosphere. This in turn may explain how these rhizobacteria are able to maintain strong associations with plants to provide protective effects, induce biomass increases and improve plant fitness. The complete lack of knowledge in the circadian biology of non-photosynthetic bacteria, combined with the potential for further understanding plant-microbe signalling, makes studying the potential circadian biology of non-photosynthetic bacteria an interesting new avenue for research.

This chapter examines the signalling of circadian timing information from host plants to root-colonising bacteria and aims to explore whether there is an influence of plant circadian rhythms on *B. subtilis* circadian oscillations. This aim is explored using several transgenic strains of *B. subtilis* kindly donated by the laboratory of Prof Martha Merrow (Ludwig Maximilian University of Munich).

The genes of interest for each strain and the function of the proteins they encode for are detailed in Table 3.1.

Gene	Function	References
resE	Sensor kinase. Part of the two-component regulatory system involved in switching to anaerobic respiration.	Nakano et al. (1996) Sun et al. (1996)
ykoW	Also known as *ddcW*. Component of the signalling pathway of the intracellular second messenger c-di-GMP that regulates adaptation process such as virulence and motility.	Gao et al. (2013)
ydfH	A sensor kinase and member of the two-component regulatory system YdfH/YdfI. Function unknown.	Serizawa & Sekiguchi (2005)
yycG	Sensor kinase. Part of the two-component regulatory system with *yycF* which regulates expression of *ftsAZ* operon. Essential for cell wall metabolism and cell division.	Fukuchi et al. (2000) Howell et al. (2003)
phoR	A histadine sensor kinase. Part of the two-component regulatory system with *phoP* to control the Pho regulon in response to phosphate starvation. Involved in regulating phosphate homeostasis, initiating sporulation and interacting with the ResD-ResE system to control respiration.	Hulett et al. (1994) Eymann et al. (1996) Birkey et al. (2002)
kinC	Histadine kinase that stimulates the phosphorylation of Spo0A to initiate sporulation.	LeDeaux & Grossman (1995) Jiang et al. (2000)
ytvA	Blue light photoreceptor with a LOV domain. Activates the σ^B-dependent general stress response under blue-light.	Akbar et al. (2001) Ávila-Pérez et al. (2006)

Table 3.1. *B. subtilis* genes investigated in this study and their functions. All genes code for proteins with PAS domains as found in many circadian components, making them interesting targets for early bacterial circadian studies.

These genes were selected as candidate reporters of circadian rhythmicity in *B. subtilis* as they encode proteins each with a per-ARNT-sim (PAS) domain. The PAS domain is found across all kingdoms of life including bacteria, fungi, plants, insects and vertebrates (Huang et al. 1993; Crosthwaite et al. 1997; King et al. 1997; Zhulin et al. 1997). Proteins containing PAS domains function as internal sensors, detecting stimuli such as oxygen, redox potential and light (Taylor & Zhulin 1999). Several PAS domain-containing proteins are found in circadian clock components across phyla, where they serve to sense the external environment and provide inputs to the clock. These proteins include the mammalian CLOCK and PERIOD2 proteins found in mice and the *Drosophila* PERIOD protein, all with 2 PAS domains each (Antoch et al. 1997; Hennig et al. 2009). In the ascomycete fungus *Neurospora crassa*, the clock-associated protein white collar-1 (WC-1), which is required for blue-light responses, possesses 2 PAS domains (Crosthwaite et al. 1997; Linden et al. 1997). Within the plant circadian clock, there are also many circadian clock components containing

PAS domains. This includes the following proteins from the ZEITLUPE (ZTL) gene family: ZTL, LKP2 (LOV kelch protein2), FKF1 (flavin-binding, kelch repeat, F box) (Nelson et al. 2000; Somers et al. 2000; Schultz et al. 2001). Since PAS domains are found in many circadian clock proteins, it is possible that the PAS-containing proteins that these genes encode for may be sensory input components within a bacterial circadian system and thus make promising candidates for initial studies like this.

All transgenic strains have the bioluminescent *luxABCDE* reporter under the control of the promoter of each of these *B. subtilis* genes. The *lux* genes for this operon have been isolated in several studies from the naturally bioluminescent marine bacteria *Vibrio harveyi* and *Vibrio fischeri* and used to make reporter constructs (Meighen 1991). The light-emitting reaction involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain fatty aldehyde (RCHO) which results in the emission of blue-green light with a peak emission at 490nm (Hastings & Gibson 1963). This reaction is as follows:

$$\mathbf{FMNH_2 + RCHO + O_2 \rightarrow FMN + H_20 + RCOOH + hv\ (490\ nm)}$$

The oxidation of the bacterial substrates is catalysed by a luciferase enzyme, which is produced by the *luxA* and *luxB* genes in this operon (Balwin et al. 1984; Engebrecht & Silverman 1984). The *luxC, luxD* and *luxE* are responsible for the production of a reductase, a transferase and a synthetase, respectively. Together the *luxCDE* operon generates the aldehydes required for the bioluminescence reaction (Boylan et al. 1989; Meighen 1991). This reporter is well suited to studies with bacteria as they remain capable of producing high levels of light at 37 °C, a routinely used bacterial growth temperature (Francis et al. 2000). An additional benefit of this reporter for research is its ability to generate a bioluminescent signal without the exogenous application of an aldehyde substrate. In this study, utilising this reporter enables *B. subtilis* promoter activity to be monitored easily by measuring light output from the transgenic strains listed above.

The aim of the work described in this chapter was to identify whether plant circadian rhythms influence oscillations in PAS-domain gene promoter activity in *B. subtilis*. Due to the novel and cutting-edge nature of this project, a significant amount of work was committed to developing reliable methods for the inoculation and imaging of the bioluminescent bacteria on the plant roots.

3.2. Method development

The following section describes the experiments conducted to develop methods for inoculating and imaging *B. subtilis* associations with *Arabidopsis* roots. The flowchart in Figure 3.1. outlines the experimental steps to establish this set of protocols. From this point forwards, *B. subtilis* strain names are abbreviated according to the following format: *gene of interest::lux* (e.g. *ytvA::lux).*

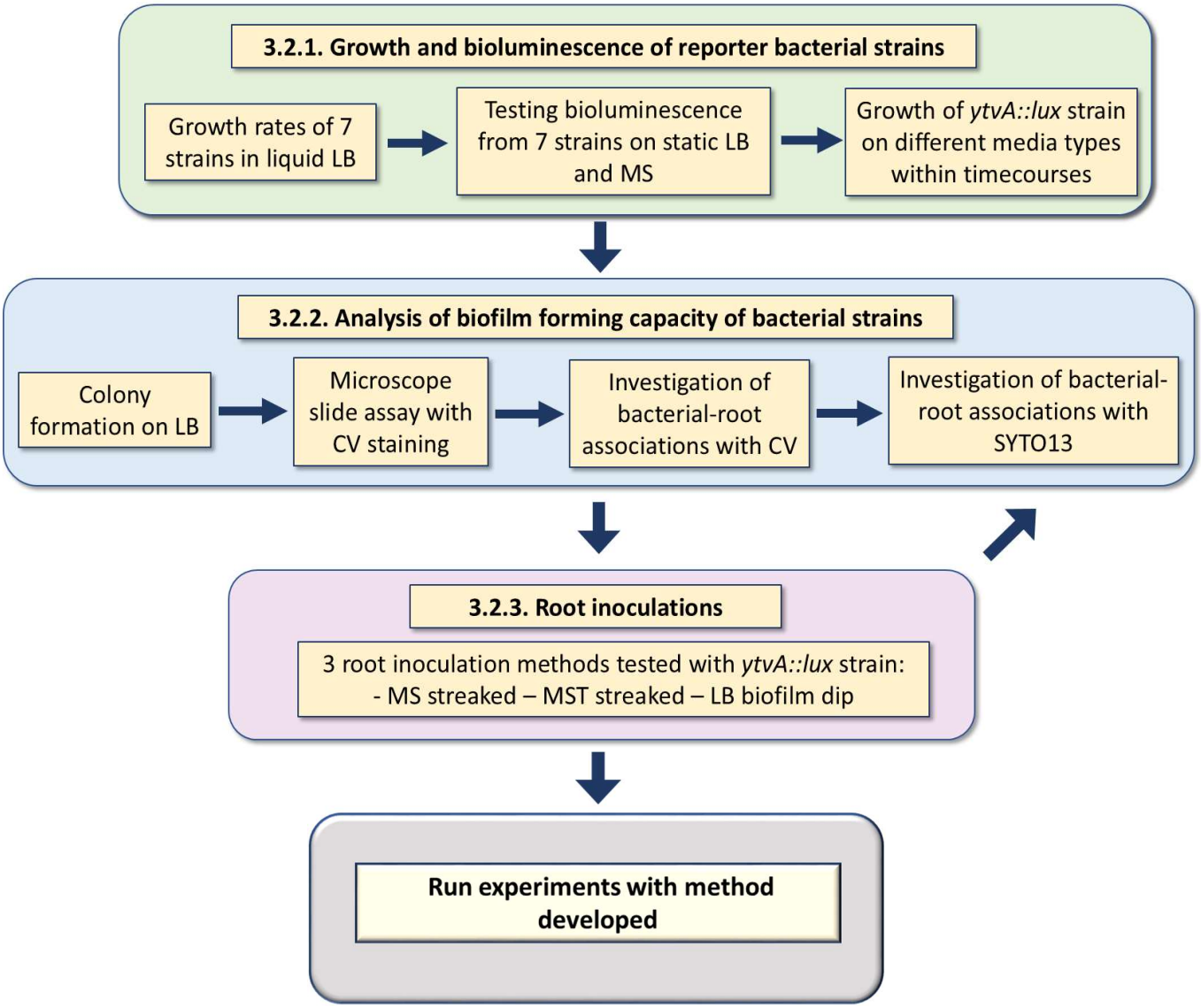

Figure 3.1. Process used to develop methods for inoculating and imaging *B. subtilis* associations with *Arabidopsis* roots. Abbreviations used are as follows. LB: Lysogeny broth. MS: Murashige & Skoog medium. MST: Murashige & Skoog medium supplemented with 1% tryptone (w/v). CV: Crystal violet.

3.2.1. Understanding growth of transformed *B. subtilis* strains and investigation of bioluminescence signal levels

First, I wanted to understand the culture conditions and growth rates for the 7 strains. This was important because I wanted to confirm that all strains would grow quickly on the bacterial growth media (LB) and produce bioluminescence at levels detectable by the ICCD camera. This would later allow cultures to be quickly generated for use as root inoculum and subsequently for the bioluminescent bacterial strains to be imaged on the roots.

3.2.1.1. Growth of transformed strains in LB liquid

Firstly, each strain was grown in liquid LB at 37 °C and the optical density was measured regularly at OD_{600}. This was to understand the growth rate of each strain in a common bacterial growth media, in this case LB. In addition, it identified the time required for the culture to reach OD_{600} 0.2-0.4, the bacterial density used in similar studies inoculating roots with *B. subtilis* (Bais et al. 2004; Beauregard et al. 2013; Allard-Massicotte et al. 2016).

As shown in Figure 3.2., these growth rate experiments revealed that all strains could be cultured in LB media. In total, the experiment was conducted 3 times. There was some variation between each replicate, however in all replicates, all strains had reached at least OD 0.2 by 4 h giving a minimum growth time for further studies in LB. Due to time constraints none of the strains reached stationary phase where cell densities plateau.

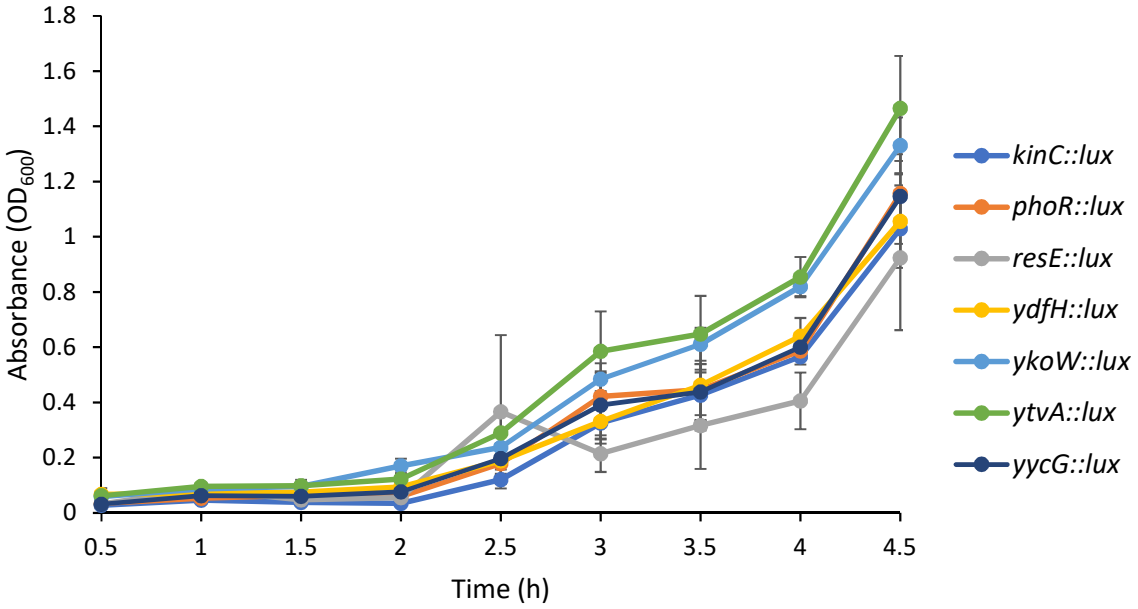

Figure 3.2. Growth of the 7 transgenic *B. subtilis* strains in liquid LB. Cell densities were measured ever 30 min using a spectrophotometer at OD_{600} (n=3). Error bars are representative of the standard error of the mean (SEM).

3.2.1.2. Growth and bioluminescence of all 7 reporter strains on solid LB and MS media

Bioluminescence imaging experiments were used to investigate whether the strains were bioluminescent, and whether they might be able to grow on and produce bioluminescence on the plant growth media (MS). The latter was important to identify the most appropriate media conditions for future experiments. All 7 strains were grown initially in LB liquid culture to OD_{600} >0.2, and a 1 ml aliquot of each culture was spread across solid LB or MS media using a sterilised glass spreader. The plates were transferred to a growth cabinet and incubated at 37 °C overnight before imaging. Images were captured using an ICCD camera, which recorded bioluminescence from the bacterial strains on the plates. Either a 0.1% or 1% virtual neutral density filter was used, according to the brightness of the bacterial strain, to ensure photon counts were maintained below 1500 counts/s. All images were captured using an integration time of 3 min.

All strains grown on LB plates produced some bioluminescence, however bioluminescence varied greatly between strains (Figure 3.3). *ykoW::lux* and *ydfH::lux* on LB both generated very low levels of bioluminescence, with under 10 counts/s with a 1% filter, which was not visible in images collected by the camera. *yycG::lux* and *ytvA::lux* generated ~40 counts/s and ~100 counts/s each. *resE::lux, kinC::lux* and *phoR::lux* on LB were all very bright, giving approximately 200 counts/s, 500 counts/s and 1000 counts/s, respectively. Photon counts from the bacteria grown on MS plates were ~10% of that of bacteria grown onto LB plates. The bioluminescence captured in these images was not observable with the naked eye so they are not included in Fig. 3.3. This reduced light output demonstrated a reduced ability for the bacteria to either grow or produce bioluminescence on MS. Therefore, it was determined that the bacteria were unlikely to grow on the MS media and produce light in the absence of plants.

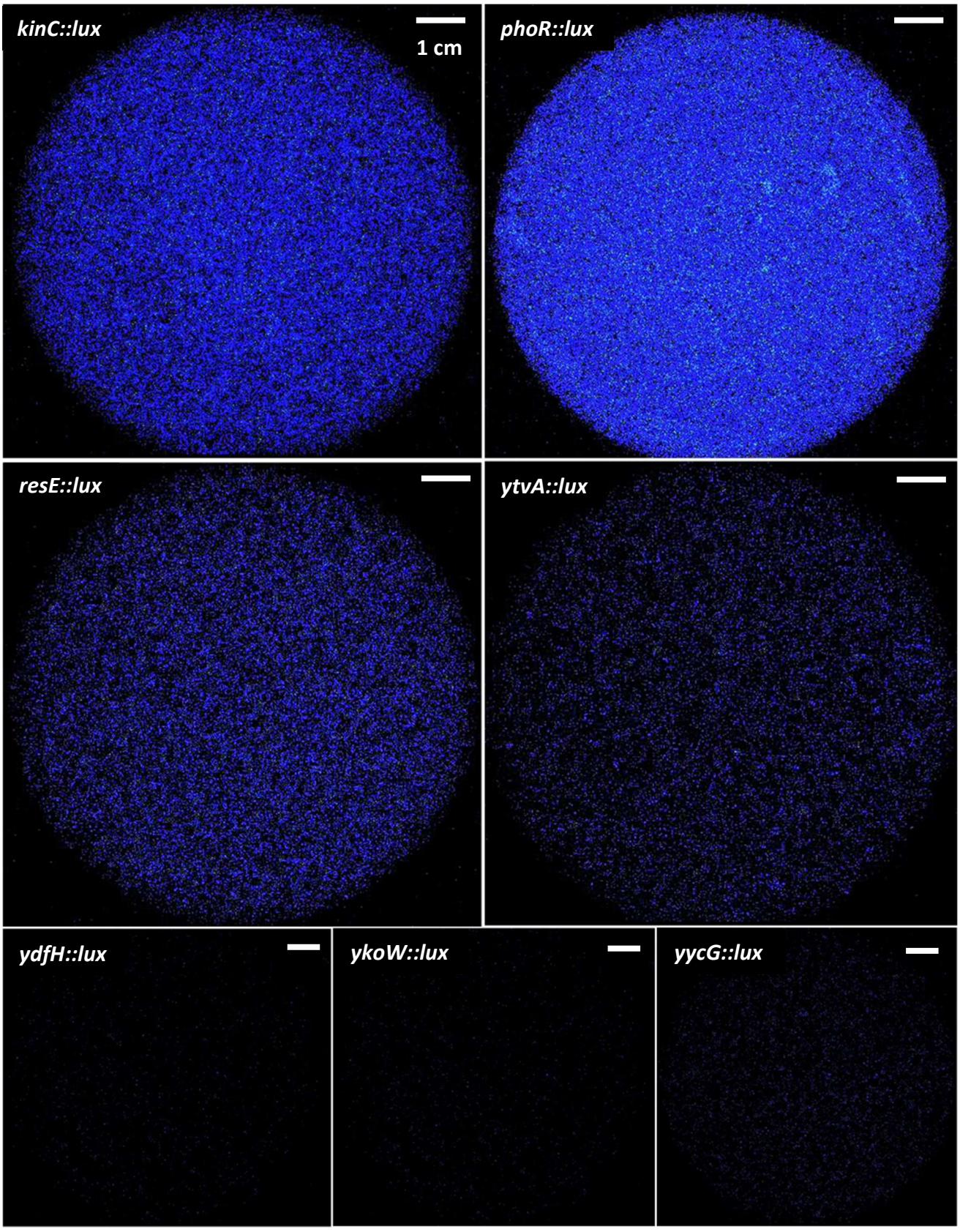

Figure 3.3. Bioluminescence output from transgenic *B. subtilis* strains on solid LB media. All strains were grown overnight at 37 °C on solid LB in 90 mm plates before imaging with an ICCD camera (n=1). Images were captured with either a 0.1% or 1% virtual neutral density filter, depending on the initial brightness. Bioluminescence signal from *ydfH::lux, ykoW::lux* and *yyCG::lux* is too low to be observed with the naked eye.

3.1.1.3. Timecourse of *ytvA::lux* bioluminescence on a variety of media types

I decided to focus upon one of the 7 strains, *ytvA::lux.* This was chosen as it had shown the most potential for circadian rhythmicity when entrained to light and temperature cycles in experiments conducted by the Merrow lab, as well as known roles in blue light sensing in *B. subtilis* (Ávila-Pérez et al. 2006). The latter gives reason to explore the possibility that YtvA is an input component in a bacterial circadian oscillator, analogous to the circadian blue light-sensing ZTL protein in the Arabidopsis circadian oscillator, which contains PAS and LOV domains like YtvA (Somers et al. 2000; Kim et al. 2007).

A timecourse experiment was used to monitor how bacterial bioluminescence varied over time under constant conditions (constant darkness at 21°C). The aim of this was to obtain an indication of how consistently bacterial bioluminescence was maintained in constant darkness and under the longer imaging conditions used in circadian timecourse experiments.

Bacterial cultures of the *ytvA::lux* strain were grown in 3 different media in a 6 well plate: solid LB (LBa), liquid LB (LBl), and liquid half-strength MS supplemented with 1% tryptone (w/v) (MST). The addition of tryptone was tested since the background 168 strain of *B. subtilis* is a tryptophan auxotroph, so I reasoned that addition of tryptone to the media may improve growth of the transgenic strain on MS (Anagnostopoulos & Crawford 1961). Bacterial cultures were first grown to stationary phase (OD_{600} > 1.5) in LB and given one of the following treatments: i) diluted 1:100 in LB and streaked onto 5 ml LBa, ii) diluted 1:100 in LB and 5 ml of this solution added per well or, iii) a 1 ml aliquot of culture centrifuged at 1500 rpm for 5 min to harvest cells, resuspended in MST, diluted 1:100 in MST and 5 ml added to well. Each media was also supplemented with the antibiotic chloramphenicol and 2 replicate wells were assigned to each media in a 6 well plate. The timecourse was initially set to run on a program taking measurements every 30 min for 24 h with a 3 min integration and a 1% virtual neutral density filter. However, by 19 h the bioluminescence was too bright and risked damaging the camera, so the time course was reset but with a 10 min integration time and a 0.1% virtual neutral density filter, running for 48 h. The results from these two timecourse analyses are in Figure 3.4.

The bacteria were able to produce bioluminescence in all 3 media treatments although there was some variability in bioluminescence. In liquid LB, the bacteria had the highest and fastest initial increase in bioluminescence, peaking at 16 h with the curve resembling a growth curve. Although, bacteria grown in MS supplemented with tryptone (MST) produced the lowest levels of light over the course of the experiment, these images were still extremely bright and demonstrated that the addition of tryptone improves growth in MS. Bioluminescence from bacteria streaked and grown on

LBa increased the slowest, but followed a similar shape curve to LBl, peaking after 22 h. This delayed increase is likely due to the differences in cell counts: the diluted *ytvA::lux* culture was streaked onto LB, meaning the initial cell number in the well was much lower than the other 2 treatments. Nevertheless, this experiment showed that under all 3 treatments, *ytvA::lux* produced consistently high bioluminescence output over the several days in darkness and under the lower temperatures of the camera box (21 °C) Therefore, *ytvA::lux* was a suitable strain for these imaging conditions.

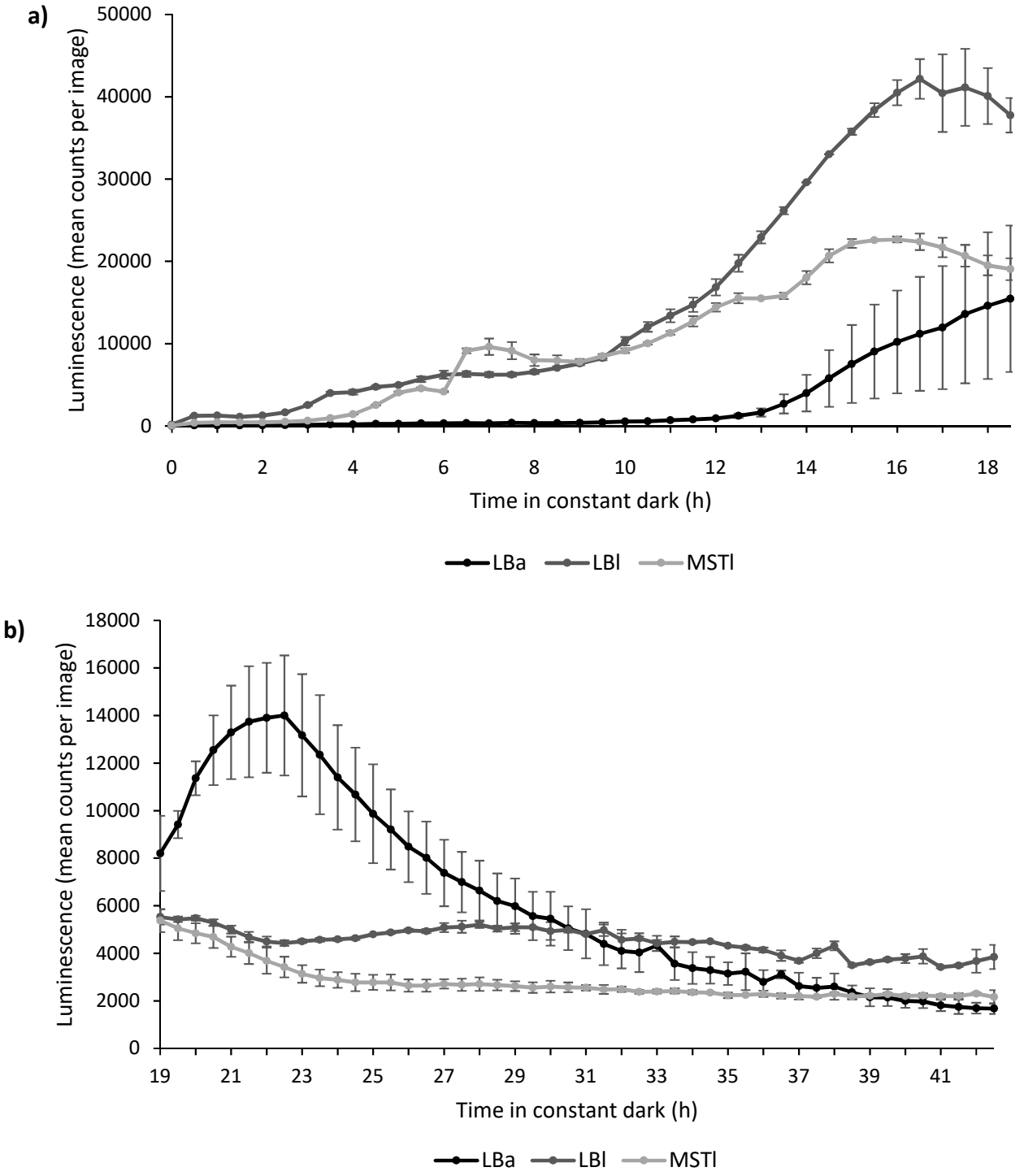

Figure 3.4. Time course experiment showing bioluminescence output of *ytvA::lux* strain grown on different media in constant darkness. LBa: LB agar. LBl: LB liquid. MSTl: MS liquid supplemented with 1% tryptone (w/v). Graphs correspond to the same plate, but the experiment was reset at 19 h with a different program to avoid high light levels damaging the camera (n=2). a) 1% virtual neutral density filter, 3 min integration every 30 min. b) 0.1% virtual neutral density filter, 10 min integration every 30 min. Error bars are representative of the standard error of the mean (SEM).

3.2.2. Investigation of biofilm formation in all transgenic reporter strains

Biofilms are communities of closely-associated microorganisms held together by an extracellular matrix of polysaccharides, proteins and DNA produced by the cells (Branda et al. 2001; Branda et al. 2006). A complex network of genes regulates the switch from free-living planktonic lifestyle to a non-motile stage which results in triggering this biofilm formation (Vlamakis et al. 2013). Biofilms are thought to provide an ecological advantage to bacteria by conferring protection against environmental stresses, increasing nutrient availability, removing toxic metabolites and assisting in the acquisition of new genetic traits via horizontal gene transfer (Davey & O'Toole 2000).

Regarding bacterial associations with plants, it is well established that biofilm formation is required for successful root colonisation by *B. subtilis* (Beauregard et al. 2013; Dietel et al. 2013). Therefore, I wanted to investigate the extent of the biofilm forming abilities in the 7 transgenic strains, especially as the domesticated 168 background strain has been suggested to be deficient in robust biofilm formation (Branda et al. 2001; McLoon et al. 2011).

3.2.2.1. Colony formation on LB

All strains were inoculated onto solid LB and incubated at 37 °C for 24 h before being examined for characteristics of biofilm formation. All strains showed some biofilm-forming characteristics such as a slight wrinkling of the colony surface and edges, due to the formation of the extracellular matrix around the cells (Figure 3.5a, 3.5b) (Vlamakis et al. 2013). Gallegos-Monterrosa et al. (2016) found similar biofilm characteristics when growing different 168 variants in LB supplemented with glucose. The 168 Jena variant, from which these transgenic strains were derived, produced opaque wrinkled colonies that resembled those formed by the undomesticated 3610 strain and was able to colonise *Arabidopsis* roots like 3610. The similarity between the biofilms produced by these transgenic colonies and the 168 and 3610 colonies documented by Gallegos-Monterrosa et al. (2016) indicate that these transgenic strains have still retained some biofilm forming capacity and could likely colonise plant roots for this study.

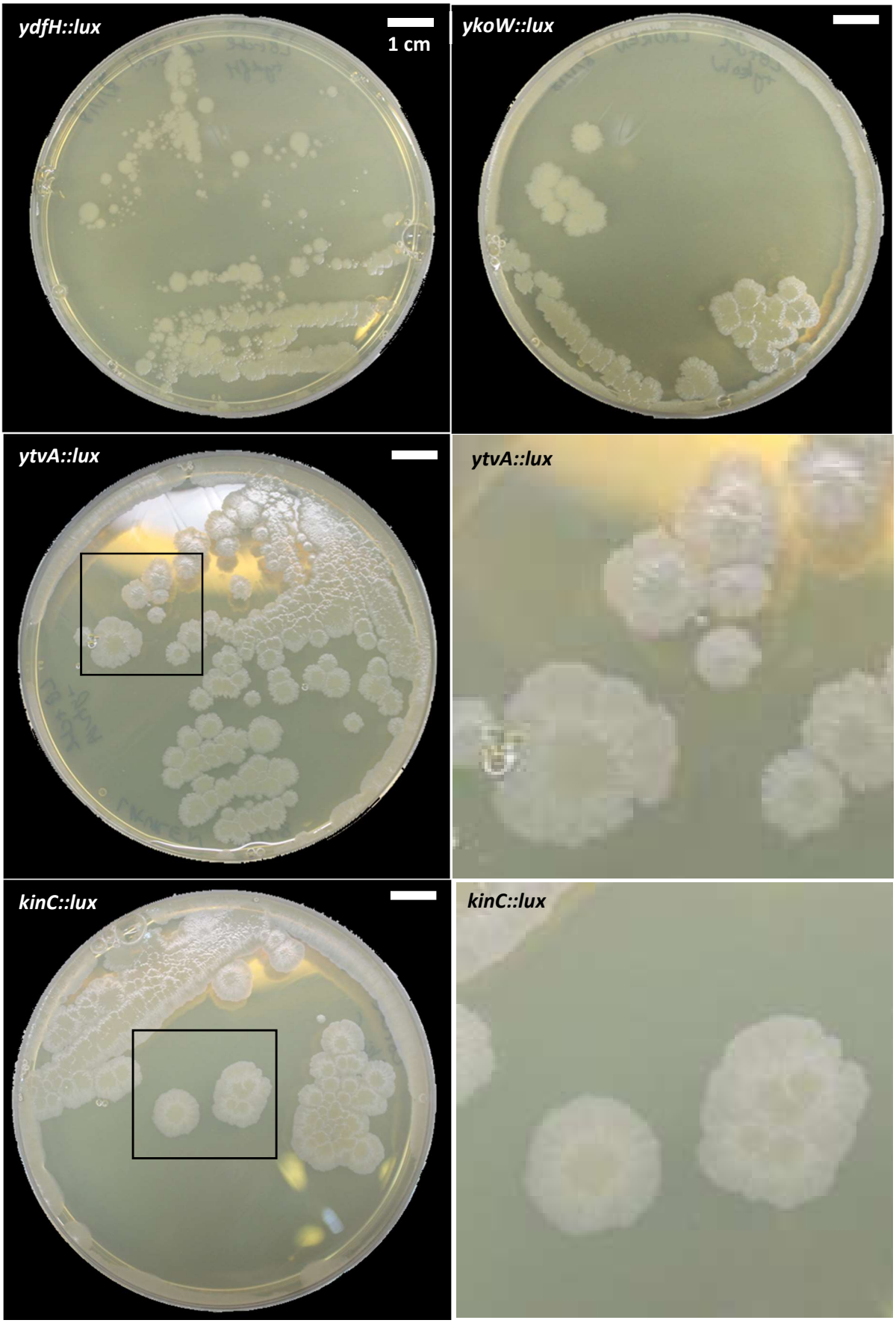

Figure 3.5a. Growth of *B. subtilis* strains on solid LB in 90 mm plates. Bacterial colonies show some indication of biofilm formation with a raised, wrinkled appearance**.**

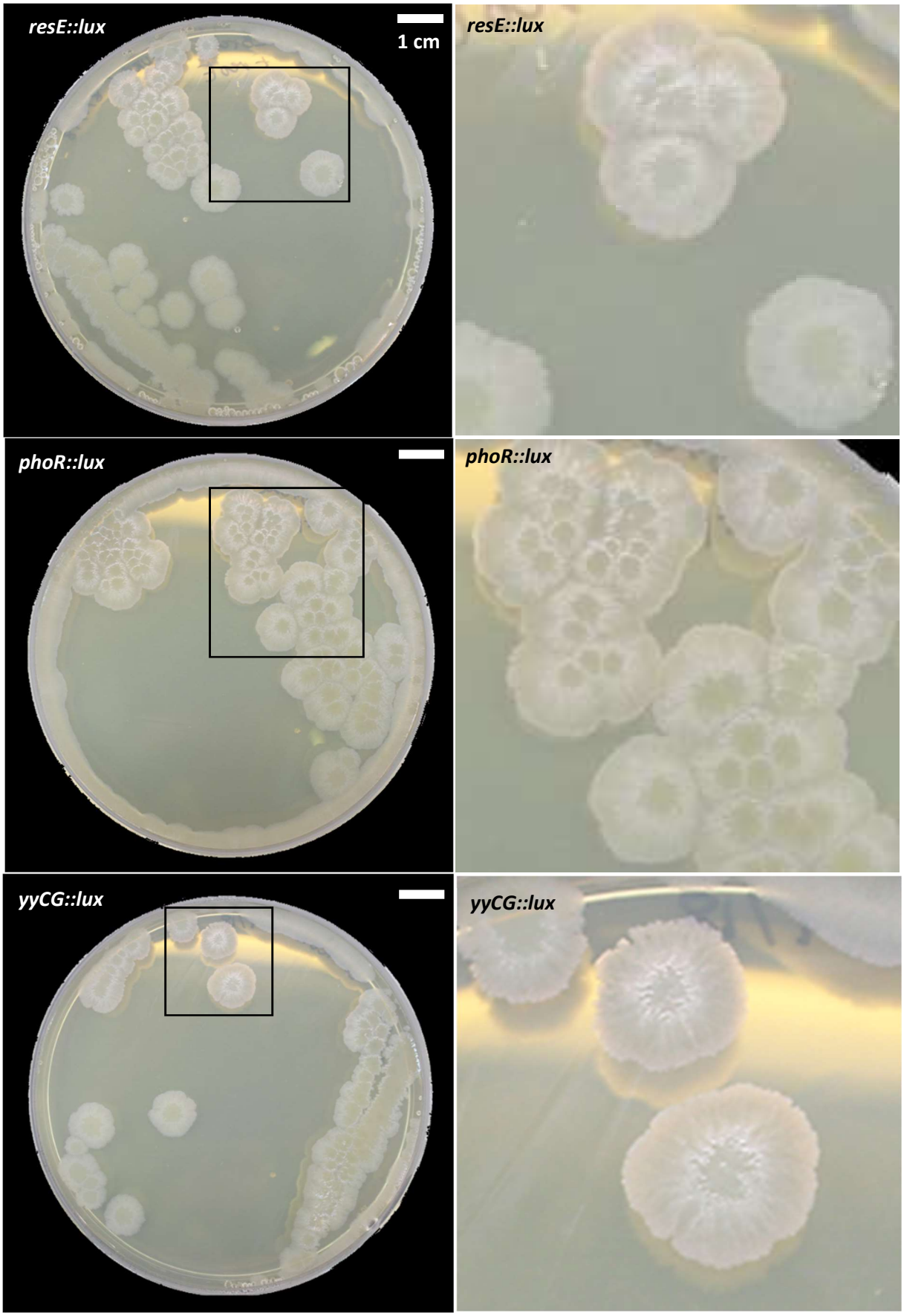

Figure 3.5b. Growth of *B. subtilis* strains on solid LB in 90 mm plates. Bacterial colonies show some indication of biofilm formation with a raised, wrinkled appearance**.**

3.2.2.2. Investigation of biofilms with crystal violet staining

Growth on LB gave some indication of biofilm formation but to confirm that these strains were genuinely producing biofilms a microscopy-based experiment was used. This used a method adapted from Merritt et al. (2005). All strains were grown at 37 °C in liquid LB in a 50 ml falcon tube containing a sterile microscope slide. The addition of the slide meant that the biofilm that formed at the air-liquid interface (known as a pellicle), would adhere to the slide. The slide was then carefully removed, the biofilm stained with crystal violet (CV) and examined with a light microscope (see 2.2.3. and 2.5.1.1. for method).

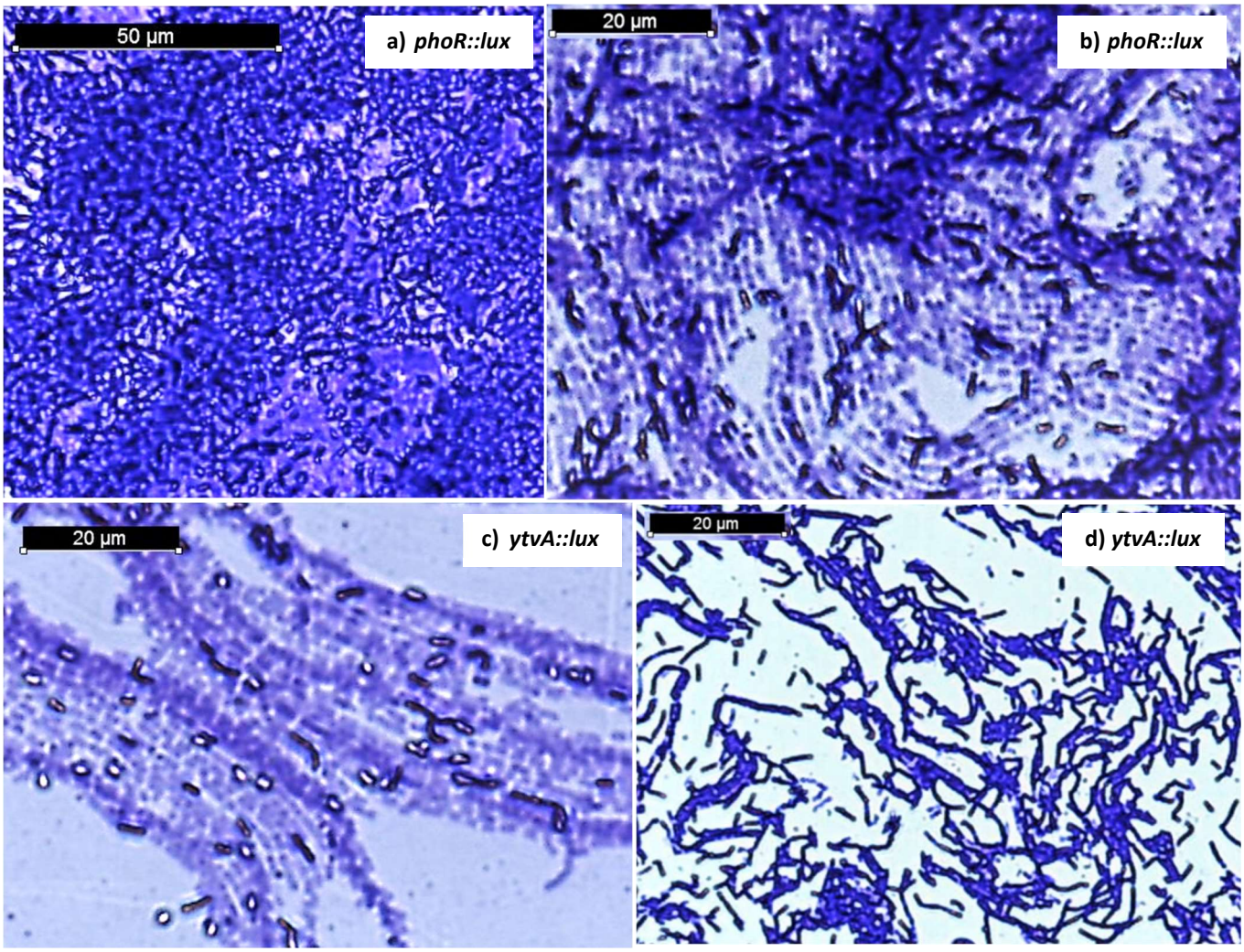

Figure 3.6. Microscope slide assay for examining crystal violet (CV) stained *B. subtilis* biofilms. All images are of biofilms grown for 24 h on a glass microscope slide before staining with 0.1% CV and imaged using a light microscope. a) *phoR::lux* strain showing aggregation of cells into large masses. b) *phoR::lux* strain showing long chains of bacilli characteristic of biofilm formation. c) *ytvA::lux* strain also showing formation of chains of bacilli. d) *ytvA::lux* strain demonstrating the disassembly of cells in chains.

Under the microscope, in all strains many of the bacterial cells had aggregated into large masses that resemble the complex aggregations in mature *B. subtilis* biofilms documented by Kobayashi (2007) (Figure 3.6a). In samples such as *phoR::lux* and *ytvA::lux*, chains of bacilli could also be easily observed (Fig. 3.6b and 3.6c). These chains are characteristic of true biofilms and represent the early to mid-stages of biofilm formation prior to the formation of complex aggregations (Kobayashi 2007; Lemon et al. 2008). It is unknown whether these chains were visible because of being separated from a more complex layered biofilm during the staining process, or whether these chains were the extent of the biofilm forming abilities in these strains. The former is perhaps more likely as the staining process broke up much of the fragile biofilms, which may have also separated the biofilm layers from each other (Fig. 3.6d). Nevertheless, the presence of these chains demonstrated *phoR::lux* and *ytvA::lux* strains to be capable of biofilm formation.

3.2.2.3. Testing biofilm formation on plant roots with crystal violet staining

Next, I wanted to test whether CV could also be used to stain *B. subtilis* biofilms on *Arabidopsis* roots to identify whether the bacteria were forming close associations with the roots. To do this, plants were inoculated by streaking the roots with a culture of *ytvA::lux* that had been resuspended and diluted in either MS only or MST and incubated for 4 days to allow for biofilm formation (Bais et al. 2004; Beauregard et al. 2013; Chen et al. 2013). This was followed by one of two CV staining methods (simple CV or with the addition of Gram's iodine) as described previously in section 2.5.1.2. These stained root samples were imaged using light microscopy, like the microscope slide-based biofilms.

The CV and Gram's iodine both stained the roots very strongly and so any biofilm that might have formed on the roots was indistinguishable from the root (Figure 3.7). In addition, the staining process appeared to remove any adhered bacteria from the root or made the bacteria unable to bind to the root initially, thus leaving bacterial cells suspended in the mounting solution. The staining process also damaged the roots, breaking off multiple root hairs. This identified that CV and the staining method used was not appropriate for staining these bacterial-root associations. It was also possible that the method for inoculating the roots with bacteria was not suitable for allowing biofilm formation. Therefore, I explored other root inoculation methods and staining methods.

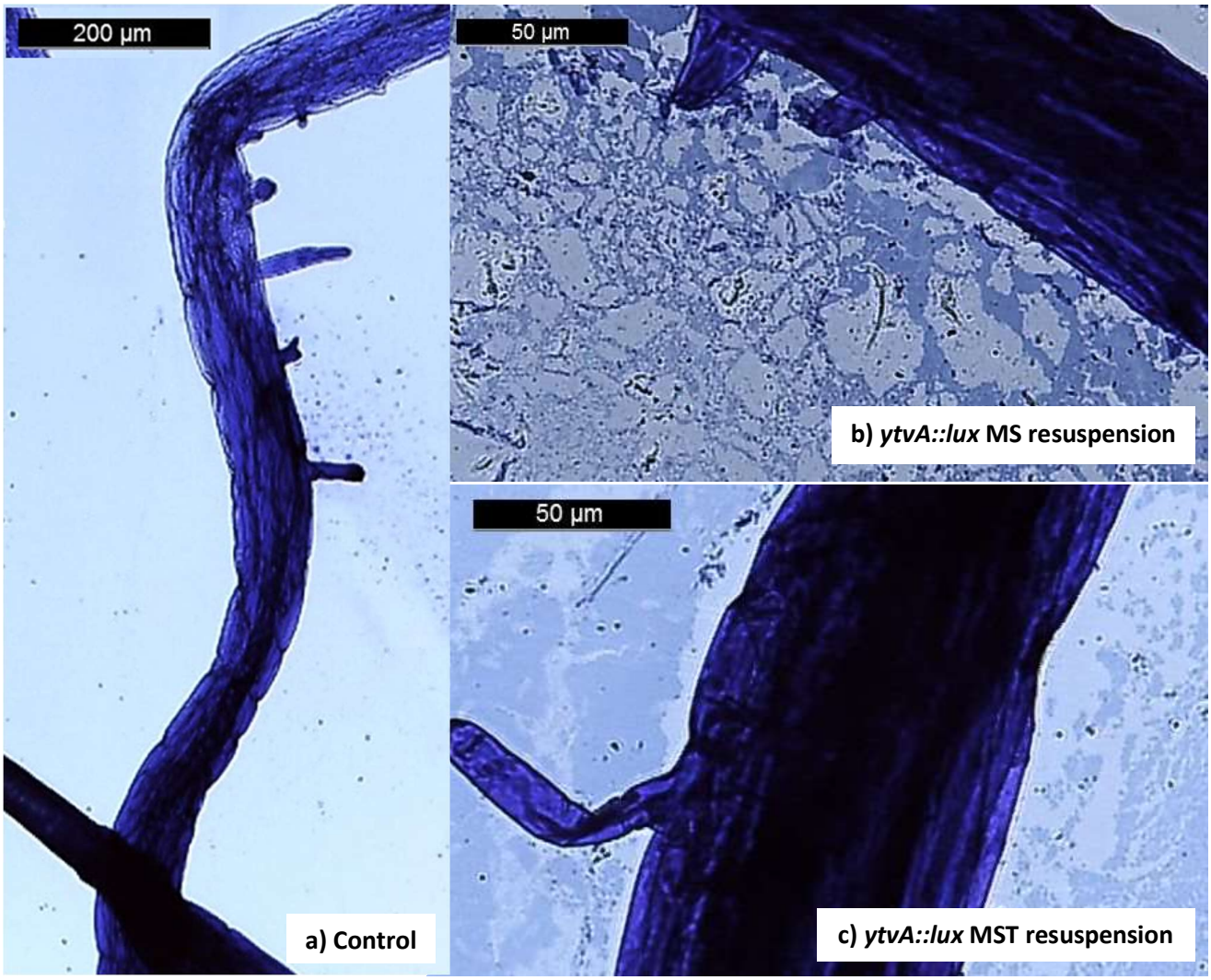

Figure 3.7. Simple crystal violet (CV) staining of *Arabidopsis* roots inoculated with *B. subtilis ytvA::lux* strain. Images are representative of 3 different root samples: a) control root treated with MS only and stained; b) root inoculated with bacteria resuspended in MS and; c) root inoculated with bacteria resuspended in MS + 1% tryptone (w/v) (MST).

3.2.2.4. Staining bacterial-root associations with SYTO13

Alongside testing methods for root inoculations (see 3.2.3), a different dye for staining *B. subtilis* on plant roots was tested. Following a literature review of suitable dyes (see Table A1 in Appendices), the fluorescent dye SYTO13™ was selected. SYTO stains have been used effectively in other studies of *Bacillus* associations with plants (Bais et al. 2004; Rudrappa et al. 2008; Kumar et al. 2012b; Soares et al. 2015). They are cell-permeant dyes that are used to stain RNA and DNA in both eukaryotic and prokaryotic cells. Upon binding they undergo a large fluorescent enhancement with bright green emission.

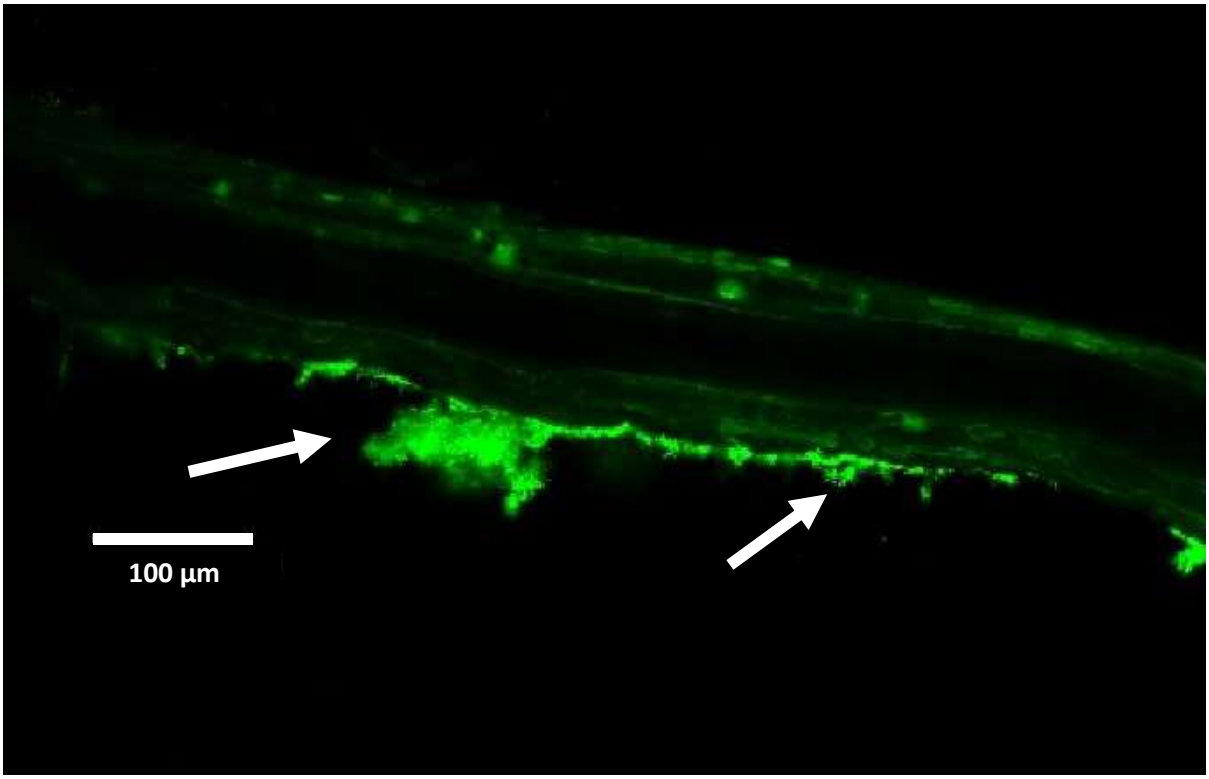

Figure 3.8. *B. subtilis* 168 biofilm formation on wild-type *Arabidopsis* root. White arrows highlight SYTO13-stained bacterial biofilms on the surface of the root. The 488 nm laser line of the argon laser was used for excitation and images were collected at 500-560 nm.

SYTO13 staining and confocal microscopy was conducted according to the protocol in 2.5.2. Wild-type plants and the non-labelled *B. subtilis* background strain were used to avoid bioluminescence from the roots or bacteria interfering with images collected by the confocal microscope.

Images revealed that the biofilm dip method (designed in 3.2.3) effectively inoculated the roots (Figure 3.8). Aggregations of *B. subtilis* formed on the outside of the roots, indicating biofilm formation and demonstrating the close association between plant roots and bacteria. The diffuse appearance of the bacterial colonies suggests the bacteria are embedded in an extracellular matrix of exopolysaccharides, indicative of a true biofilm (Davey & O'Toole 2000).

3.2.3. Root treatments

I wished to determine best method for inoculating *Arabidopsis* that would enable the growth of *B. subtilis* biofilms with consistent bioluminescence on roots.

Methods

Three different inoculation treatments were used: for two of these, the *ytvA::lux* strain was grown in liquid culture until the cell density reached stationary phase (OD_{600} > 1.5). Then a 1 ml aliquot of this culture was centrifuged at 1500 rpm for 5 min and either resuspended and diluted 1:100 in liquid MS or liquid MST to generate the inocula. The third inoculum was produced by culturing *ytvA::lux* in a 6 well plate. 7 ml of LB was added per well and inoculated with a colony taken from a previously inoculated LB plate. The 6-well plate was incubated at 37 °C without shaking for 48 h until a biofilm had formed at the air-liquid interface.

For root inoculations, 5 ml of static half-strength MS was added to each well of a 6 well plate and each treatment was designated 2 wells each (Figure 3.9). In 4 of these wells, nine 20-day-old *Arabidopsis* plants were placed across the surface of the MS in each well so that their roots were spread out forming a bed. This enabled the MS and MST cultures to be spread across their roots using an inoculation loop. For the 2 remaining wells, plant roots were carefully dipped into the biofilm that had formed in the non-shaking *ytvA::lux* cultures to coat the roots in bacteria. This method of dipping roots has been used in other studies inoculating plant roots with *Bacillus* (Fan et al. 2012; Dietel et al. 2013). The plants were then positioned across the surface of the MS, ensuring the roots were visible. Again, 9 plants were inoculated per well. The plate with these inoculated plants was placed under the ICCD camera 2 h post-inoculation and images were taken every 30 min with a 0.1% virtual neutral density filter and an integration time of 10 min over the course of 3 days. After 48 h the program was stopped and reset with a 10% virtual neutral density filter and an integration time of 10 min for 2 days. This was done due to the light output from MST roots reaching extremely high photon counts which may have masked light output from neighbouring samples.

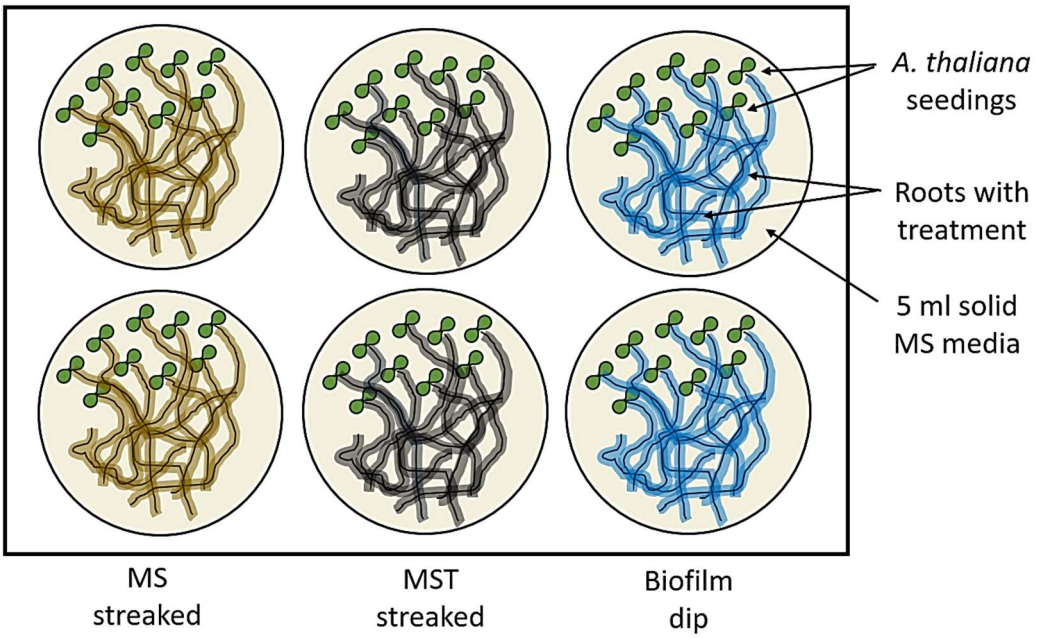

Figure 3.9. Arrangement of inoculated *Arabidopsis* roots in a 6-well plate. Nine 20-day-old plants per well with roots positioned across the surface of a 5 ml solid MS base. Roots inoculated with *ytvA::lux* resuspended in MS (yellow) or MST (MS supplemented with tryptone; black) by streaking with an inoculation loop, or by dipping roots in a culture of bacteria that was grown previously for 2 days in liquid LB (biofilm dip; blue).

Results

As mentioned above, the wells with the MST treatment were removed after 48 h as the roots were too bright for successful imaging of other treatments and the bacteria appeared to have grown on the solid MS base, perhaps using the tryptone in the liquid solution (Figure 3.10). Although clearly giving high bioluminescence output, this treatment was deemed not appropriate as the bacteria were not growing solely on the roots.

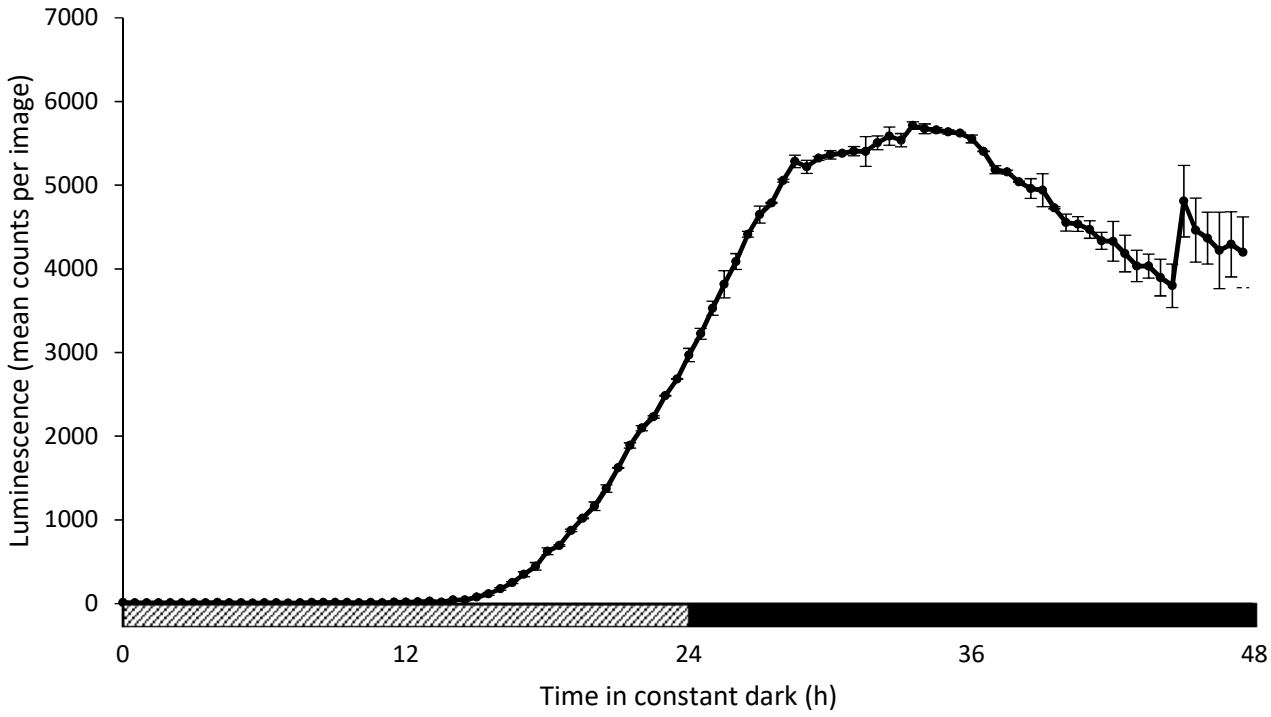

Figure 3.10. *B. subtilis ytvA::lux* activity from the bacteria in the MST treatment on roots. Bacteria were grown to stationary phase, then resuspended and diluted in MS supplemented with 1% tryptone (w/v) and streaked across roots. Images taken every 30 min over 48 h with a 0.1% virtual neutral density filter and an integration time of 10 min (n=2). Hatched and solid black boxes represent subjective light and dark periods for the host plant. Error bars are representative of the standard error of the mean (SEM).

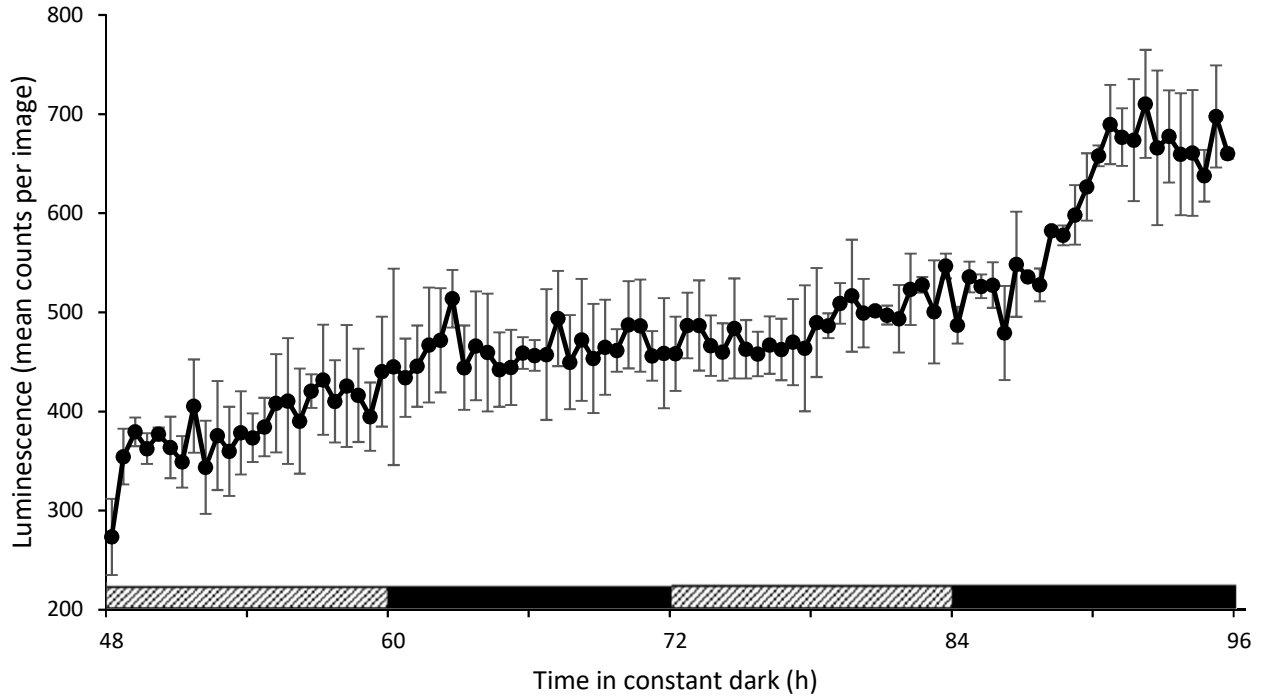

Figure 3.11. *B. subtilis ytvA::lux* activity from the bacteria in MS treatment on roots. Bacteria grown as with the MST treatment but resuspended and diluted in MS only and streaked across roots. Images taken every 30 min over 48 h with a 10% virtual neutral density filter and an integration time of 10 min (n=2). Time is displayed relative to the start of the initial timecourse program. Hatched and solid black boxes represent subjective light and dark periods for the host plant. Error bars are representative of the standard error of the mean (SEM).

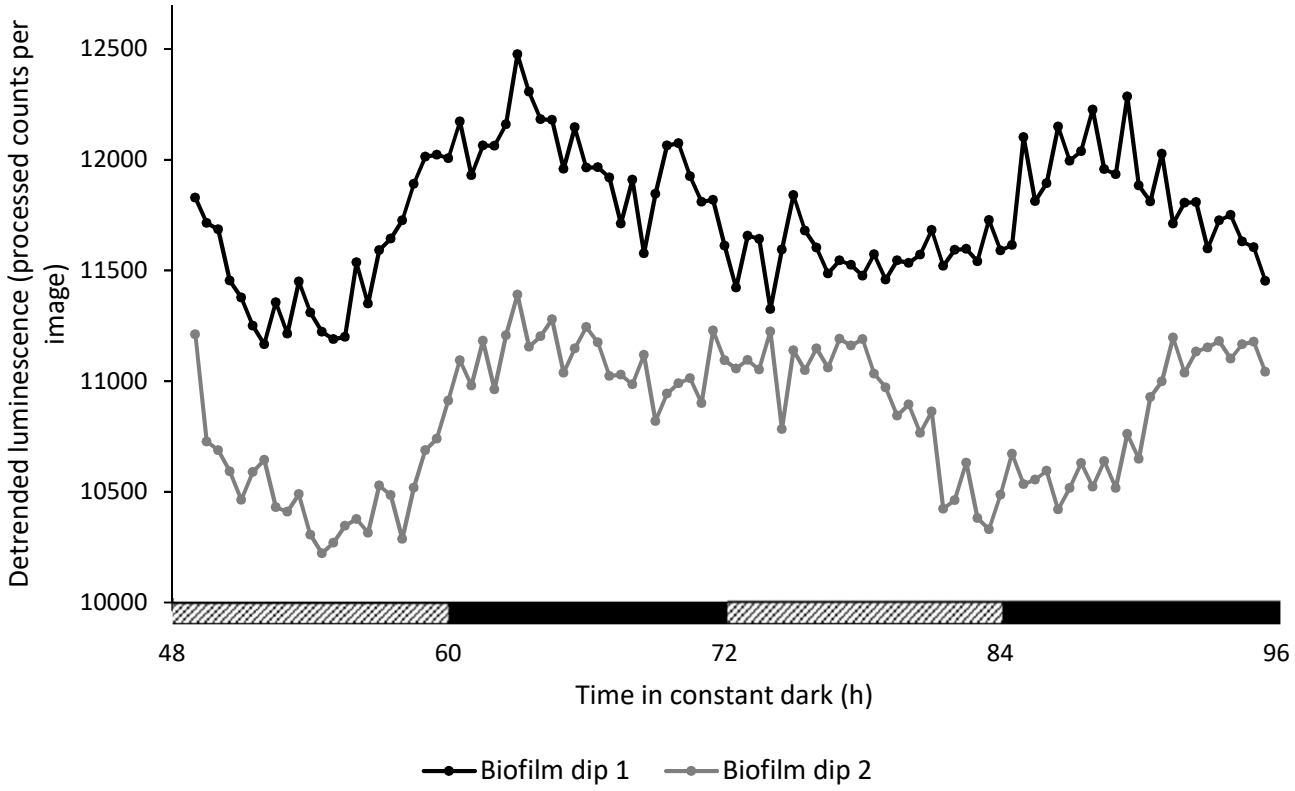

Figure 3.12. *B. subtilis ytvA::lux* activity from bacteria in biofilm dip treatment on roots. Roots were dipped in the biofilm that had formed on the surface of a static culture of *ytvA::lux* grown for 48 h. Time is displayed relative to the start of the initial timecourse program. Images were captured every 30 min with an integration time of 10 min using a 10% virtual neutral density filter. Data were detrended using amplitude and baseline detrending. The wells are plotted individually to visualise the potential rhythmicity in the biofilm dip sample 1 (n=1). Hatched and solid black boxes represent subjective light and dark periods for the host plant.

Figures 3.11 and 3.12 show the bioluminescence output from the transgenic bacteria in the MS and biofilm dip treatments, respectively. The first hour has been omitted as the light output was much higher. This may have resulted from exposure to light in the room when the camera dark box was opened to remove the MST treatment wells. Bacteria that were resuspended in MS and streaked onto roots had significantly reduced light output compared to the bacteria from the biofilm culture on roots (peaked at 764.75 counts per image for MST compared to 12844 counts per image for biofilm dip). This difference may be the result of large differences in cell density: the MS inoculum was a very diluted form of the bacteria with a cell density of OD_{600} 0.015, whereas the biofilm dip culture was undiluted and had a cell density of $OD_{600} > 2.5$. Another issue with the MS treatment was that the liquid culture spread across the surface of the solid MS media when applied, rather than adhering to the plant roots. Therefore, it was decided to disregard this MS treatment for future inoculations.

Biofilm dip bacteria produced consistently high levels of bioluminescence on the roots over the course of the experiment (Figure 3.12 and Figure 3.13). Interestingly, the bioluminescence appears to be rhythmic for bacteria in one of the wells (biofilm dip 1), perhaps suggesting any bacterial circadian rhythms are conditional upon unknown factors. To analyse this dataset for rhythmic features a Fast Fourier Transform Non-linear Least Squares (FFT NLLS) analysis was run using BioDare2 (www.biodare2.ed.ac.uk, Nov. 2018). The bacteria in biofilm dip 1 had a period of 23.34 h, close to the 24 h period of the host plant. However, this is a very small sample size and the other biofilm dip treatment (biofilm dip 2) did not have rhythmicity with a period of approximately 24 h. In addition, the duration of the time series was only 2 days and it is recommended to analyse a minimum of 2.5 days for determining whether data is circadian or not (Zielinski et al. 2014). When periodicity was tested in BioDare2 using a Maximum Entropy Spectral Analysis (MESA) analysis, neither biofilm sample exhibited circadian rhythmicity so whether this was a true circadian rhythm in *B. subtilis* remains inconclusive. Nevertheless, the potential rhythmicity in one of the wells alongside high bioluminescence output from bacteria on these biofilm-coated roots provided the evidence that this was the best root inoculation method.

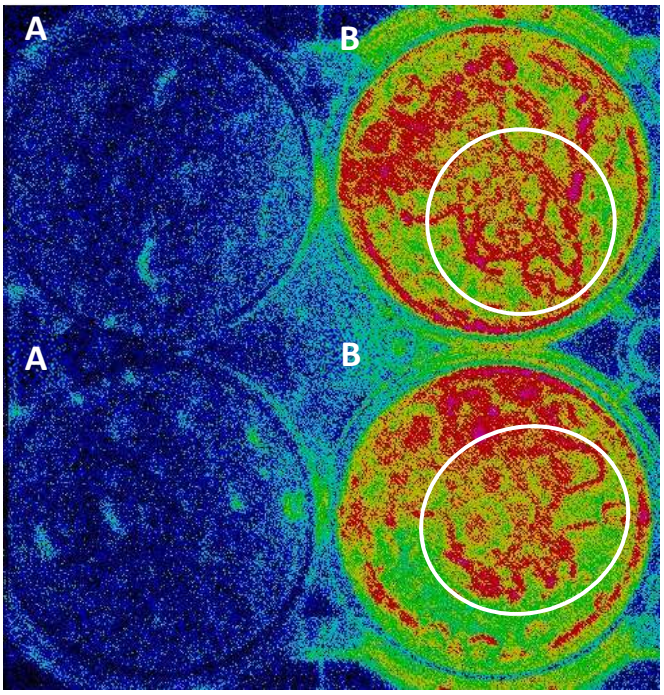

Figure 3.13. Bioluminescence image of *ytvA::lux B. subtilis* on *Arabidopsis* roots. MS streaked treatment (A) presents very low levels of light. Biofilm dip treatment (B) gives high levels of bioluminescence from bacteria coating roots (circled). Greatest bioluminescence intensities are shown in red, lowest in blue.

3.2.4. Summary of method development

This method development gave an understanding of several physiological properties of the *B. subtilis ytvA::lux* strain that allowed a complete protocol to be designed. Firstly, the transgenic *B. subtilis* grew quickly in liquid LB media which would allow for the rapid generation of inoculum (4 h minimum). Growth on solid LB and staining with crystal violet identified that *ytvA::lux* can form biofilms, as indicated by the formation of chains of bacilli. Subsequent staining of inoculated roots with the fluorescent dye SYTO13 showed *B. subtilis* forms close associations with *Arabidopsis* roots. Timecourse experiments revealed the bacteria produced consistently high bioluminescence on the roots over the course of several days following a biofilm dip treatment, demonstrating that a root dip was the most suitable inoculation method for yielding consistently high light output.

3.3. Effect of *Arabidopsis* circadian rhythms on *ytvA* promoter activity

The aim of the work described in this chapter was to identify whether plant circadian rhythms influence circadian oscillations in *B. subtilis* (Figure 3.14). Following the development of a method for inoculating and imaging the roots, experiments were conducted to explore this potential for signalling of circadian timing information from the plant to the *ytvA::lux* strain of *B. subtilis.*

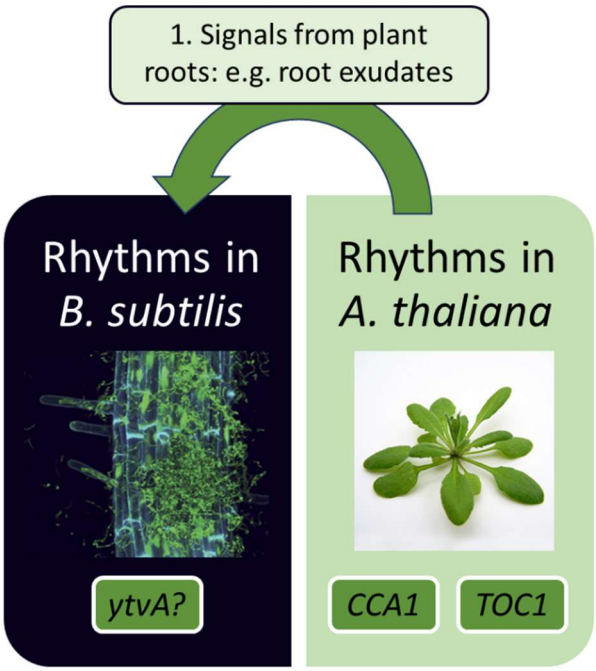

Figure 3.14. Model for the hypothesised signalling of circadian timing information from *A. thaliana* host plants to *B. subtilis*. White bordered rectangles represent some core circadian clock genes in plants (*CCA1* and *TOC1*) and potential genes in the putative bacterial circadian system (*ytvA*). Labelled arrow indicates signal which may influence circadian rhythms in the symbiotic bacteria.

To test this, plants were grown under 2 opposing sets of lighting regimes and inoculated using the biofilm dip developed during the method development. It was hypothesised that if the bacteria were able to be entrained by temporal signals from the plant roots (such as root exudates) then they would obtain the same rhythm of their host plant. This would generate 2 sets of bacteria with rhythms of *ytvA* promoter activity that had a period of 24 h but were in antiphase with each other (peaking 12 h apart).

3.3.1. Methods

For plant growth, both sets of plants were grown under 12 h:12 h light:dark cycles but with one set illuminated from 9am to 9pm, termed the forward-entrained, and one set lit from 9pm to 9am, termed the reverse-entrained. This generated the 2 sets of host plants with opposing circadian rhythms. These plants were inoculated with bacteria at least 11 days after germination by dipping the roots in a culture of *ytvA::lux* that had been grown for 48 h in a 6 well plate (the biofilm dip). 6 inoculated plants were carefully transferred per well in a 6-well plate, with their roots laid out forming a bed (Figure 3.15). For the control, 2 wells contained plants that had their roots dipped in LB. Plates were placed under the ICCD photon counting camera and images were collected every 30 min with an integration time of 10 min and a 10% virtual neutral density over the course of 7 days. This timecourse of bioluminescence imaging was conducted under constant darkness.

Figure 3.15. Experimental configuration for studying effects of plant circadian rhythm on *ytvA* promoter activity. 1 and 2: forward-entrained and reverse-entrained control plants respectively, with roots dipped in LB. 3 and 4: forward-entrained plants with roots treated with *ytvA::lux* biofilm dip. 5 and 6: reverse-entrained plants with roots treated with *ytvA* biofilm dip. 6 plants per well on 5 ml solid MS.

3.3.2. Results

The 4 independent repeats of this experiment are shown separately as each produced different results.

3.3.2.1. Replicate 1 (R1)

Figure 3.16 summarises the results of the first timecourse experiment, showing the changes in *ytvA* promoter activity as measured by monitoring bioluminescence from the transgenic *B. subtilis* over time. The first 96 h show no circadian effects, with bioluminescence from the bacteria gradually decreasing. However, *ytvA* promoter activity begins to cycle rhythmically following 96 h, which can be seen clearly when the dataset was shortened and detrended using amplitude and baseline detrending in BioDare2. Circadian properties of this dataset were analysed from 96 h onwards using FFT NLLS analysis with amplitude and baseline detrending and it was found that *ytvA* promoter activity cycled with a period of approximately 24 h for both treatments: 23.48 h ± 0.37 for bacteria on forward-entrained plants versus 24.21 h ± 0.02 for bacteria on reverse-entrained plants. Relative amplitude error (RAE) values for FFT NLLS analysis ranged from 0.21-0.33. RAE values give an indication of rhythm robustness, where values closest to 0 are robust and close to 1 are weak (or no) rhythmicity (Plautz et al. 1997; Zielinski et al. 2014). Therefore, these values below 0.5 indicate robust circadian rhythms. When MESA analysis was used to test for circadian oscillations, the analysis yielded similar period estimates with 23.47 h ± 0.45 for the forward-entrained treatment and 23.85 h ± 0.13 for the reverse-entrained treatment. RAE values ranged from 0.52-0.75. Although these values are higher than with FFT NLLS, they still demonstrate statistically significant circadian rhythmicity (RAE values below 1) and show the bacteria exhibit at least weak circadian oscillations in *ytvA* promoter activity (Plautz et al. 1997).

Both sets of bacteria had *ytvA* oscillations that appeared to be circadian regulated for these 3 days, but they were not in antiphase with each other as would be expected if perfectly entrained to the host plant. Bioluminescence from bacteria on forward-entrained plants peaked at 22.19 h ± 0.03, compared to 18.39 h ± 0.04 for bacteria on reverse-entrained plants. Although there was a slight difference in phase between the 2 groups, a t-test could not be used to test for significant differences as the sample size was too small to meet the conditions required (n=2 per treatment). When this was repeated with MESA analysis phase estimates were closer: 22.26 h ± 9.93 compared to 20.45 h ± 0.575 for bacteria on forward-entrained or reverse-entrained plants.

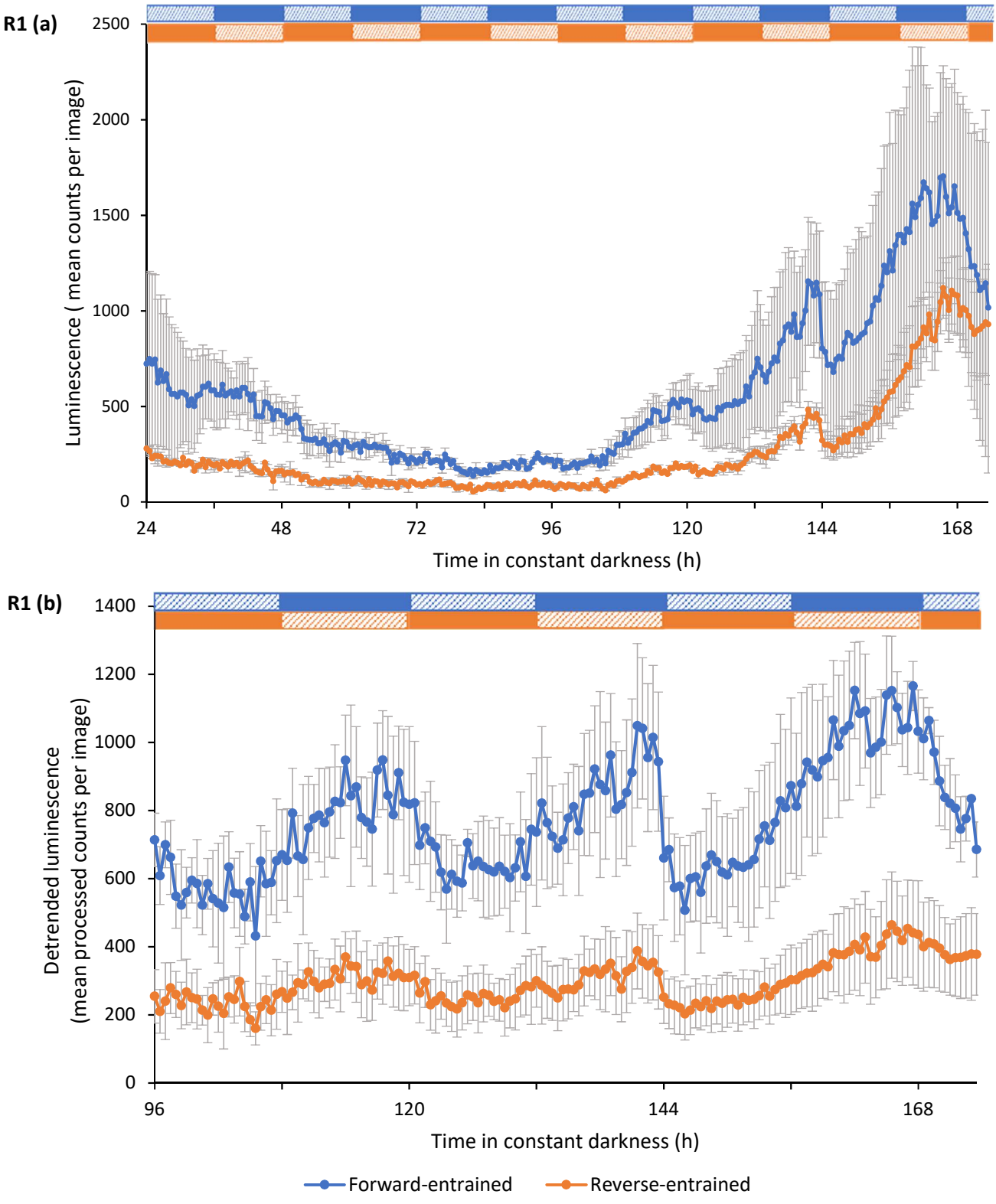

Figure 3.16. Replicate 1: *B. subtilis ytvA::lux* activity under constant darkness. Bioluminescence from bacteria on forward-entrained plants (blue) and reverse-entrained plants (orange) (n=2). Images were collected by the ICCD photon counting camera every 30 min, with an integration time of 10 min and a 10% virtual neutral density filter. Error bars represent the standard error of the mean (SEM). a) Raw data from full timecourse. b) Portion of timecourse data (96 - 173.5 h) detrended with amplitude and baseline detrending. Blue hatched and solid bars represent subjective light and dark periods for forward-entrained host plants, orange bars represent the same but for reverse-entrained plants.

3.3.2.2. Replicate 2 (R2)

When the experiment was repeated, there was a pronounced reduction in bioluminescence from bacteria on both forward-entrained and reverse-entrained plants over time (Figure 3.17a). The large decreases in bioluminescence were likely due to the failure of the temperature sensor connected to the photon counting camera. This resulted in the plate being held at approximately 8 °C instead of 21 °C. Nevertheless, the data appeared to be rhythmic and oscillations were clearly visible when the data was detrended with amplitude and baseline detrending (Figure 3.17b). FFT NLLS generated period estimates of 24.87 h ± 0.22 for bacteria in the forward-entrained treatment and 24.61 h ± 0.07 for bacteria in the reverse-entrained treatment. In contrast to the first replicate, phase estimates were much lower: 13.36 h ± 1.15 and 14.30 h ± 0.34 for bacteria on forward-entrained and reverse-entrained plants, respectively. RAE values ranged from 0.32-0.42 suggesting robust circadian rhythms. This suggests some robustness to temperature fluctuations. However, when this was repeated with MESA analysis, RAE values indicated the data were not circadian (1 for both treatments).

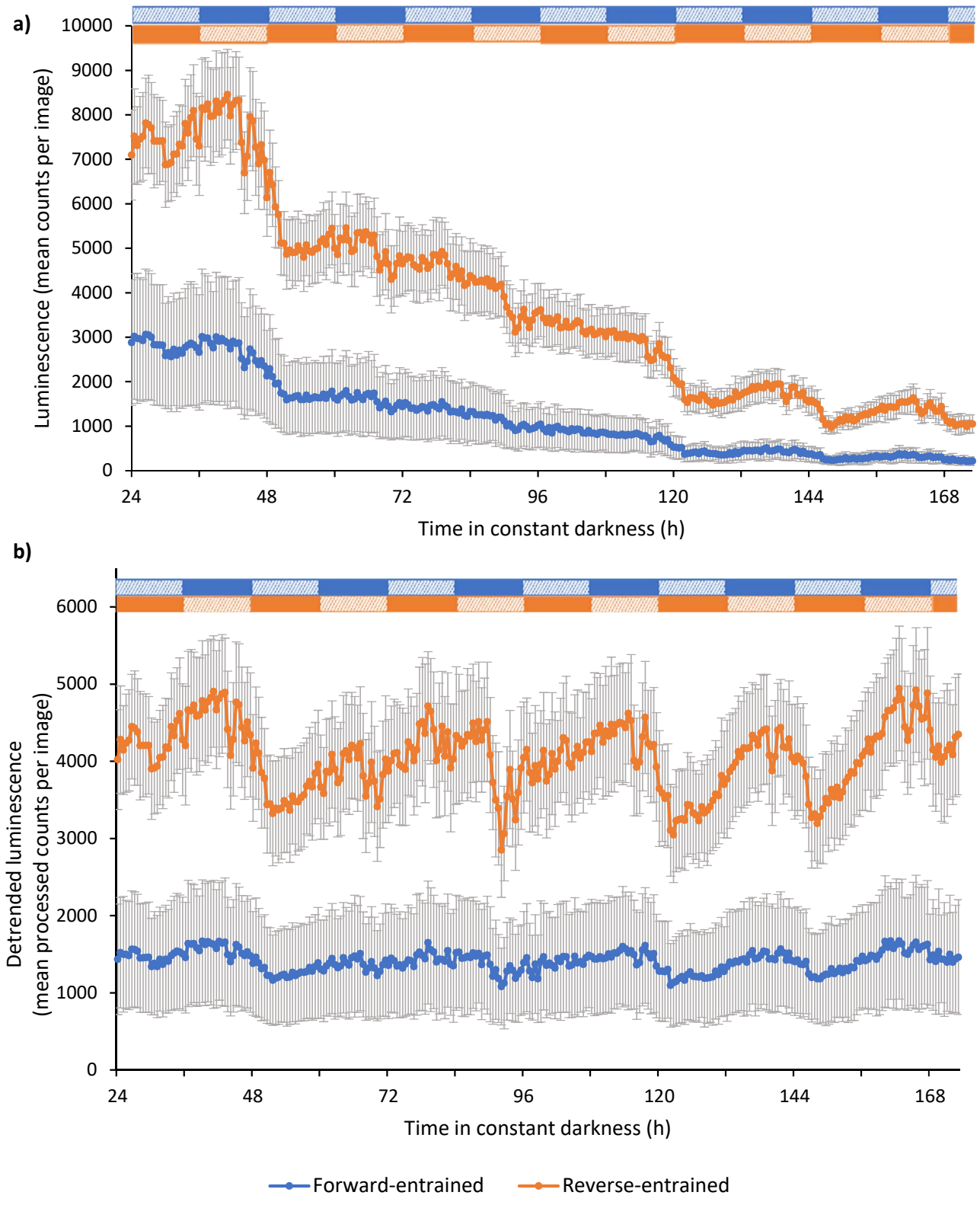

Figure 3.17. Replicate 2: Rhythms of *ytvA::lux* activity in constant darkness. Bioluminescence from bacteria on forward-entrained plants (blue) and reverse-entrained plants (orange). Images were collected by the ICCD photon counting camera every 30 min, with an integration time of 10 min and a 10% virtual neutral density filter (n=2). a) Raw data b) Data detrended using amplitude and baseline detrending. Error bars represent the standard error of the mean (SEM). Blue hatched and solid bars represent subjective light and dark periods for forward-entrained plants, orange bars represent the same but for reverse-entrained plants.

3.3.2.4. Replicates 3 & 4 (R3 & R4)

Finally, replicates 3 and 4 yielded similar results to each other but these did not provide evidence for circadian regulation (Figure 3.18). The bioluminescence for these replicates had a very different temporal pattern to the previous replicates, with the graph forming a smooth curve that rose gradually after 48 h and then decreased before plateauing.

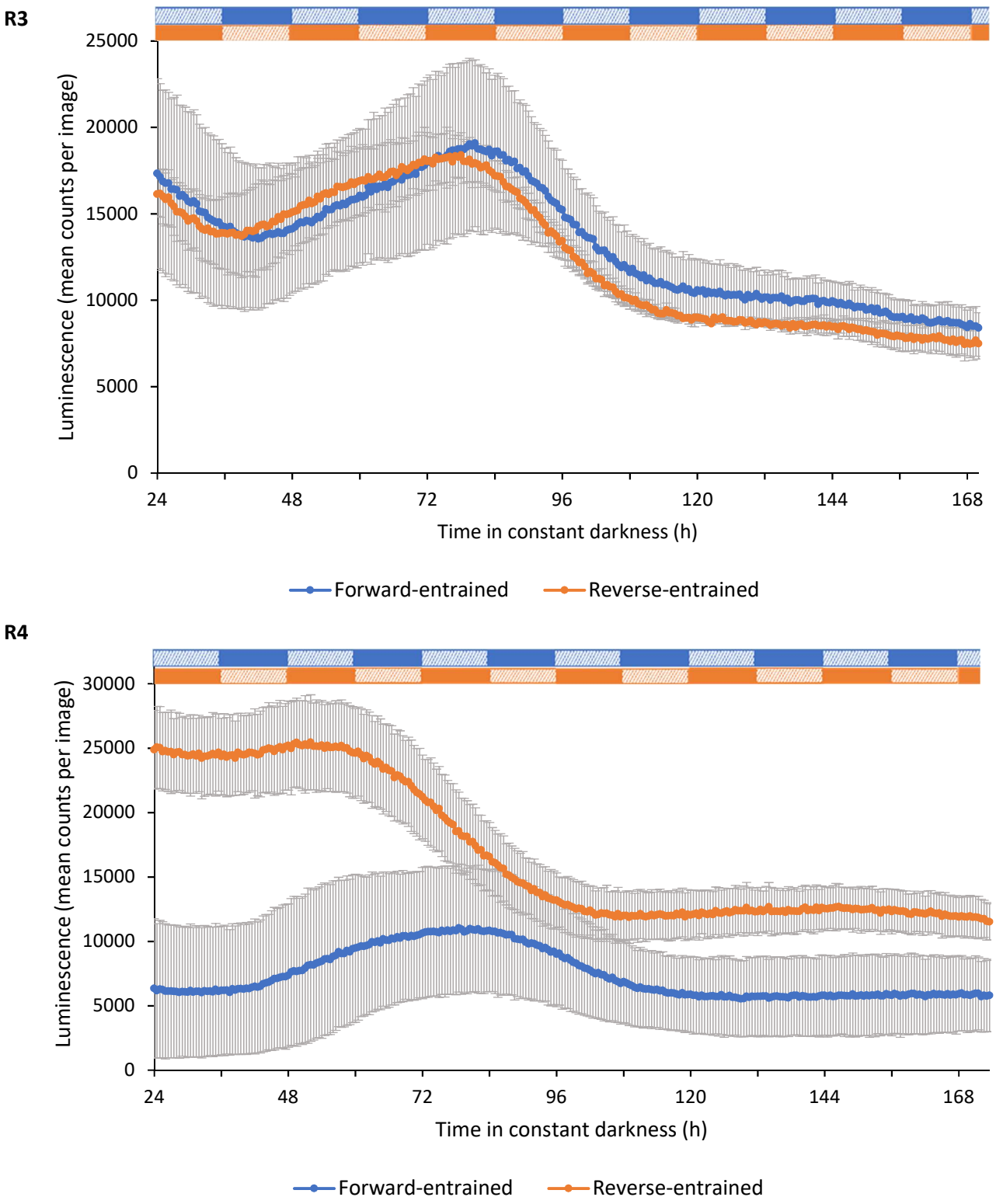

Figure 3.18. Replicate 3 (top) and replicate 4 (bottom): Rhythms of *ytvA::lux* activity in constant darkness. Bioluminescence from bacteria on forward-entrained plants (blue) and reverse-entrained plants (orange). Images were collected by the ICCD photon counting camera every 30 min, with an integration time of 10 min and a 10% virtual neutral density filter (n=2). Error bars represent the standard error of the mean (SEM). Blue hatched and solid bars represent subjective light and dark periods for forward-entrained plants, orange bars represent the same but for reverse-entrained plants.

3.3.3. Summary of the effect of *Arabidopsis* rhythms on *ytvA* promoter activity

Table 3.2 summarises the results of these 4 replicates, showing period and phase estimates alongside differences observed in the growth of the biofilm dip used for root inoculations and differences in timecourse imaging conditions.

Replicate	Forward-entrained period estimate	Reverse-entrained period estimate	Forward-entrained phase estimate	Reverse-entrained phase estimate	Inoculum growth	Timecourse imaging conditions
1	23.48 h ± 0.37	24.21 h ± 0.02	22.19 h ± 0.03	18.39 h ± 0.04	48 h, 37 °C, not wrapped	DD, 21 °C constant
2	24.87 h ± 0.22	24.61 h ± 0.07	13.36 h ± 1.14	14.30 h ± 0.34	48 h, 37 °C, wrapped in foil	DD, 8 °C (faulty sensor)
3	No rhythmicity detected.	No rhythmicity detected.	No rhythmicity detected.	No rhythmicity detected.	48 h, 37 °C, foil wrapped – reduced biofilm formation	DD, 21 °C constant
4	No rhythmicity detected.	No rhythmicity detected.	No rhythmicity detected.	No rhythmicity detected.	48 h, 37 °C, foil wrapped – reduced biofilm formation	DD, 21 °C constant

Table 3.2. Summary of results for the effect of *Arabidopsis* host rhythms on *B. subtilis ytvA* promoter activity. Period and phase estimates are reported for *ytvA::lux* activity in bacteria on forward-entrained or reverse-entrained plants. Only results from FFT NLLS analysis are given. Inoculum growth refers to the conditions, and any additional observations made, during the generation of the biofilm dip used for root inoculations. All bioluminescence timecourse imaging was conducted in constant darkness (DD), with inadvertent changes to imaging temperature due to a faulty temperature sensor.

3.4. Discussion

3.4.1. Potential effects of plant circadian rhythms on *B. subtilis ytvA* promoter activity

The objective of this chapter was to investigate the influence of plant circadian rhythms on *B. subtilis ytvA* oscillations. This would build on the evidence for an entrainable circadian system in a non-photosynthetic bacterium, following other studies which have reported on periodicity in growth of bacterial colonies (Thompson et al. 1995; Soriano et al. 2010; Paulose et al. 2016). I hypothesised that the *B. subtilis* strains would become synchronised to the rhythm of the host plant, by using cues such as root exudates to entrain a hypothetical bacterial circadian oscillator. Experimentally, this

would result in 2 sets of *B. subtilis* with *ytvA* promoter activity oscillating with a period of 24 h but in antiphase (peaking 12 h apart).

There was variation in the patterns of *ytvA* promoter activity between experimental repeats (Table 3.2). Of most interest is R1 and R2 which, when tested with FFT NLLS analysis, showed evidence of circadian rhythms with a period of 24 h for bacteria on both forward-entrained and reverse-entrained plants. In R1, the oscillations observed could be the result of the experimental methods used. For this replicate the bacteria were not wrapped in foil when cultured for the biofilm dip, therefore, the culture may have been influenced by lighting conditions in the lab. Nevertheless, this does not account for the fact oscillations did not begin at the start of the timecourse, and instead were not observed until after approximately 96 h. This delayed rhythmicity could suggest the bacteria are taking several days to become entrained to cues from the plant, such as by using root exudates. Although the 2 sets of bacteria were not in antiphase with each other, the *ytvA* promoter activity in bacteria on reverse entrained plants had an earlier phase than when on forward-entrained plants: 22.19 h ± 0.03, compared to 18.39 h ± 0.04. This suggests different entrainment effects from the oppositely-entrained sets of host plants.

Different results were obtained from R2, with *ytvA* promoter activity oscillating with a period of 24 h from the start of the timecourse. Here the possibility of entrainment prior to root inoculations is excluded as the bacteria were grown at a constant temperature and kept under constant darkness prior to inoculation. Phase estimates were advanced for this replicate in comparison with the first and bacteria on reverse-entrained plants had a delayed phase: 13.36 h ± 1.15 compared to 14.30 h ± 0.34 h. Although RAE values obtained from FFT NLLS analysis were below 0.5 (suggesting robust circadian rhythms), it should also be noted that when circadian analysis for R2 was repeated with a MESA test, the dataset was shown to be not statistically circadian. This puts doubt on whether the rhythmicity identified by FFT NLLS in this replicate is true.

The differences in the mean period between R1 and R2 (23.84 h for R1, 24.74 h for R2) could be due to differences in plant ages. In R1, plants were 22 d at inoculation. This was altered to 16 d for R2 as the plants had begun to look stressed by the end of the timecourse for R1. Circadian period is approximately 1 h shorter in older leaves than younger leaves, thus assuming these results are consistent with roots, bacteria entrained to older plants would have a shorter circadian period (Kim et al. 2016).

The differences in phase estimates between R1 and R2 could be due to possible light exposure during the plant's subjective dark period or a result of the colder timecourse conditions in R2. Reverse-entrained plants may have been exposed to a short pulse of light (<15 min) during the

inoculation process prior to imaging. It is well established that a light pulse during the subjective night can alter the phase of the plant circadian clock (Johnson 1992). The extent to which the plant phase is shifted depends on when the light pulse occurred respective to the onset of darkness, with weaker phase shifts occurring in the middle of the day and strong shifts occurring during the night (Devlin & Kay 2001). The reverse-entrained plants may have been exposed to light early on during the subjective night (<4 h), which could have resulted in the plant phase advancing by 6 h. However, the light pulses used in experiments by Devlin & Kay (2001) were 3 h long, whereas these plants were only exposed to light for a maximum of 15 min which is not likely to induce phase shifts of the same extent (Johnson 1992). In addition, phase shifts do not account for the differences in bacterial *ytvA* phase on forward-entrained plants between replicates, as these plants would have been exposed during subjective morning where phase-shift effects are limited.

The large decreases in *ytvA::lux* bioluminescence for the timecourse experiments in R2 can be attributed to the misfunctioning temperature sensor which resulted in the plate being cooled to approximately 8 °C, thus slowing metabolic activity in the bacteria and decreasing luminescence from the bioluminescent reaction (Dorn et al. 2003). Cooling of the plant plate may also explain the differences observed in phase estimates between R1 and R2. Bieniawka et al. (2008) tested the effects of chilling (4 °C) on the expression of circadian clock components and found cycles of clock output genes, such as *CCR1, CCR2, CAB2* and *CAT3*, became arrhythmic under continuous light. These conditions are similar to the conditions experienced by plants in replicate 2 (8 °C and constant darkness). Investigations in other plant species, such as chestnut (*Castanea sativa*), showed that winter dormancy disrupted the cycling of the central oscillatory components *TOC1* and *LHY* (Ramos et al. 2005). Therefore, it is likely the plant circadian oscillator was not functioning correctly in R2 and so the bacteria on the roots were not receiving the same entrainment cues as in R1. This could account for differences identified in the phase of *ytvA::lux* oscillations in R2 compared to R1.

Replicates 3 and 4 had very different temporal patterns of *B. subtilis ytvA* promoter activity on both sets of plants, with the curves closer resembling a slow bacterial growth curve. The biofilm dip used for root inoculations in these replicates did not appear to have the same extent of pellicle formation at the air-liquid interface, so perhaps the colonies that these dips were made from had reduced growth or biofilm-forming ability. This could have hindered the bacterial association with plant roots and affected the signalling between them, potentially also limiting the signalling of circadian timing information.

It is also important to consider that, for all replicates, timecourse imaging was conducted under constant darkness (DD). In these conditions, plant clock genes are regulated differently compared to

constant light. *ELF3* and the clock output gene *CCR2* have been shown to oscillate normally under continuous darkness (Covington et al. 2001; Más et al. 2003b). However, Millar et al. (1995b) found that *CAB2* promoter activity damped rapidly and cycled with a period of 30-36 h in DD compared to 24.7 h in LL. This is logical as *CAB* transcription is positively regulated by light signals (Anderson & Kay 1995). The core oscillatory component *CCA1* also has altered expression in DD, with cycles completely dampened after the first cycle (Wang & Tobin 1998). The differential expression of several plant components in constant darkness could affect the functioning of the output pathways that may be entraining the root-colonising bacteria. This means the bacteria are likely being entrained differently to what would be observed in natural conditions. Future experiments should consider keeping the plant shoots in constant light when imaging to maintain normal plant circadian function, and keeping *ytvA::lux* bacteria on the roots in darkness to mimic bacterial growth conditions in the soil and avoid accidentally activating light-responsive YtvA.

In conclusion, these experiments identified some evidence for rhythmicity in *ytvA* promoter activity entrained to opposing host plant rhythms. Results were inconsistent with differences in phase estimates between rhythmic samples and some replicates reporting no rhythmicity at all. These differences can potentially be attributed to several external conditions that may have altered the host plant rhythms. It should also be considered that these the bacterial circadian system is conditional, only occurring when the bacteria are in a complex community with integrated signalling networks and different cell types, such as in a biofilm (Vlamakis et al. 2008; Cairns et al. 2014). Activation of a circadian system could also require certain metabolites from the plant. This would account for the lack of rhythmicity in bacteria that did not form biofilms correctly on the roots. Nevertheless, the results of R1 and R2 in this chapter provide the important evidence for circadian rhythmicity in gene expression in a non-photosynthetic bacterium.

Chapter 4: Impacts of root colonisation by *B. subtilis* on circadian rhythms in *A. thaliana* shoots.

4.1. Introduction and aims

B. subtilis readily colonises roots of a range of crops including beet, carrot, radish, tea bushes, wheat and tomato (Pandey & Palni 1997; Germida et al. 1998; Fall et al. 2004; Qiao et al. 2017). As discussed previously, colonisation provides a benefit to host plants via growth enhancement and reduction in disease caused by several plant pathogens. Signalling from bacteria to the plant is an important element in eliciting these beneficial responses (Bais et al. 2004; Rudrappa et al. 2008; Fousia et al. 2015; Kobayashi 2015).

Plant growth enhancement may be achieved by secretion of phytohormones such as indole acetic acid (IAA), cytokinins, and gibberellins from *Bacillus* (Idris et al. 2004; Arkhipova et al. 2005; Gutiérrez-Mañero et al. 2008; Tahir et al 2017). Additionally, *B. subtilis* has been shown to increase the synthesis of plant chemicals, such as spermidine, promoting plant growth (Xie et al. 2014).

Colonisation by *Bacillus* can protect plants against pathogens through induced systemic resistance (ISR). Signals from the bacteria activate several defence pathways in the plant mediated by jasmonic acid (JA), ethylene (ET) and salicyclic acid (SA). This results in the upregulation of plant defence genes and leads to a number of changes such as increased production of antimicrobial phytoalexins, thickening of the cell wall, accelerated stomatal closure and increased levels of resistance-related enzymes such as peroxidase and polyphenol oxidase (Kloepper et al. 2004; Ongena et al. 2005; Nakkeeran et al. 2006; Wu et al. 2012; Fousia et al. 2015).

Clearly, *B. subtilis* has significant impacts on plant gene expression that lead to increased plant growth and disease resistance. Colonisation of plants by *B. subtilis* might also affect plant circadian clock gene expression, potentially changing the period or phase of clock genes. This signalling could be mediated by rhythmic secretion of bacterial metabolites that would act as circadian entrainment cues or other modifiers of the plant circadian clock. Alterations to the plant circadian clock that better synchronise the circadian period to that of the environment, enhance plant fitness (Dodd et al. 2005). Therefore, understanding how root-colonising bacteria influence plant circadian biology could improve our understanding of how rhizobacteria elicit improved plant growth and health.

This chapter aims to examine the signalling of circadian timing information from bacteria to host plants by investigating the impacts of root colonisation by *B. subtilis* on circadian rhythms in *A. thaliana* (Figure 4.1).

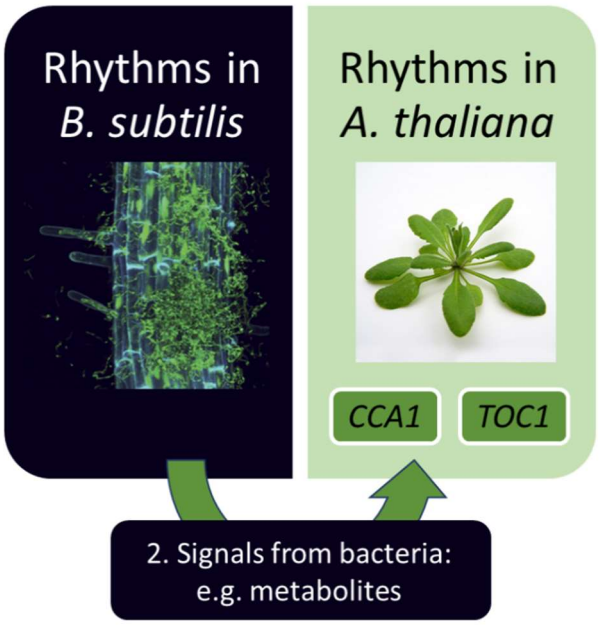

Figure 4.1. Model for the hypothesised signalling of circadian timing information from root-colonising *B. subtilis* to *A. thaliana*. White bordered rectangles represent known circadian clock genes in plants (*CCA1* and *TOC1*). The labelled arrow suggests signals that might influence circadian rhythms in the host plant.

Methodology

This investigation involved inoculating plants with the non-bioluminescent domesticated *B. subtilis* 168 strain that had previously been grown under different entrainment regimes: no entrainment (growth at constant temperature and darkness), forward-entrainment (12 h:12 h light:dark cycles lit 9am to 9pm) or reverse-entrainment (12 h:12 h light:dark lit 9pm-9am). All plants were grown under the same light regime as forward-entrained bacteria. In contrast to using bioluminescent transgenic bacteria, transgenic clock reporter plants were used to measure plant circadian rhythms. These transgenic clock reporter plants have the firefly luciferase reporter attached to the promoter element of the circadian clock genes *CCA1* or *TOC1.* This reporter allows circadian clock-associated gene expression to be monitored by measuring bioluminescence output from the plants. For both clock genes, they have an oscillation with a period of approximately 24 h, with *CCA1* peak expression occurring in the morning and *TOC1* expression peaking in the evening (Wang & Tobin 1998; Strayer et al. 2000). Therefore, the effects of inoculating with different bacterial treatments on plant circadian function are examined by analysing differences in the phase and period of these genes.

Firefly (*Photinus pyralis*) luciferase reporters, such as the reporter used in these transgenic plants, provide a simple non-invasive method for monitoring gene expression *in vivo*. The reporter gene generates the luciferase enzyme which, on addition of an exogenous luciferin substrate and in the presence of oxygen, Mg^{2+} and ATP, undergoes a reaction to produce oxyluciferin, CO_2 and a photon of light (Ow et al. 1986; Wilson & Hastings 1998). By placing this reporter gene under the control of circadian clock gene promoters, circadian rhythms of promoter activity can easily be monitored by measuring bioluminescence from the plant using a photon-counting camera. This system is frequently used to monitor the expression of circadian genes using timecourse imaging (Millar et al. 1992; Millar et al. 1995; Hall & Brown 2007; Noordally et al. 2013). In contract to the bacterial *lux* reporter system, this luciferin-based assay only requires the translation of one protein (the luciferase) with the substrate and other reagents supplied exogenously.

4.2. Results

4.2.1. Influence of manipulation on plant circadian rhythms

I wished to determine whether the method I had developed for inoculating plants with *B. subtilis* (see 3.2.3) was disrupting their circadian rhythm. This was important to ensure that any effects of inoculation were only due to *B. subtilis* or its potential circadian rhythms. I identified a series of steps in this procedure and systematically tested whether they caused alterations in the circadian rhythm in *Arabidopsis.*

CCA1::LUC and *TOC1::LUC* plants were grown on vertical petri dishes until 11 days old. Three plants were removed per six on a plant plate and replaced with plants transferred from another MS plate, mimicking the physical manipulation that occurs during the inoculation process (Figure 4.2). These plants were each treated with 100 µl 10 mM luciferin and imaged using a photon counting camera. The program captured images every 30 min with a 5 min integration and a 1% virtual neutral density filter for 4 days. In total, 2 replicates were conducted generating 6 samples per treatment: *CCA1::LUC* unmoved, *CCA1::LUC* moved, *TOC1::LUC* unmoved, *TOC1::LUC* moved.

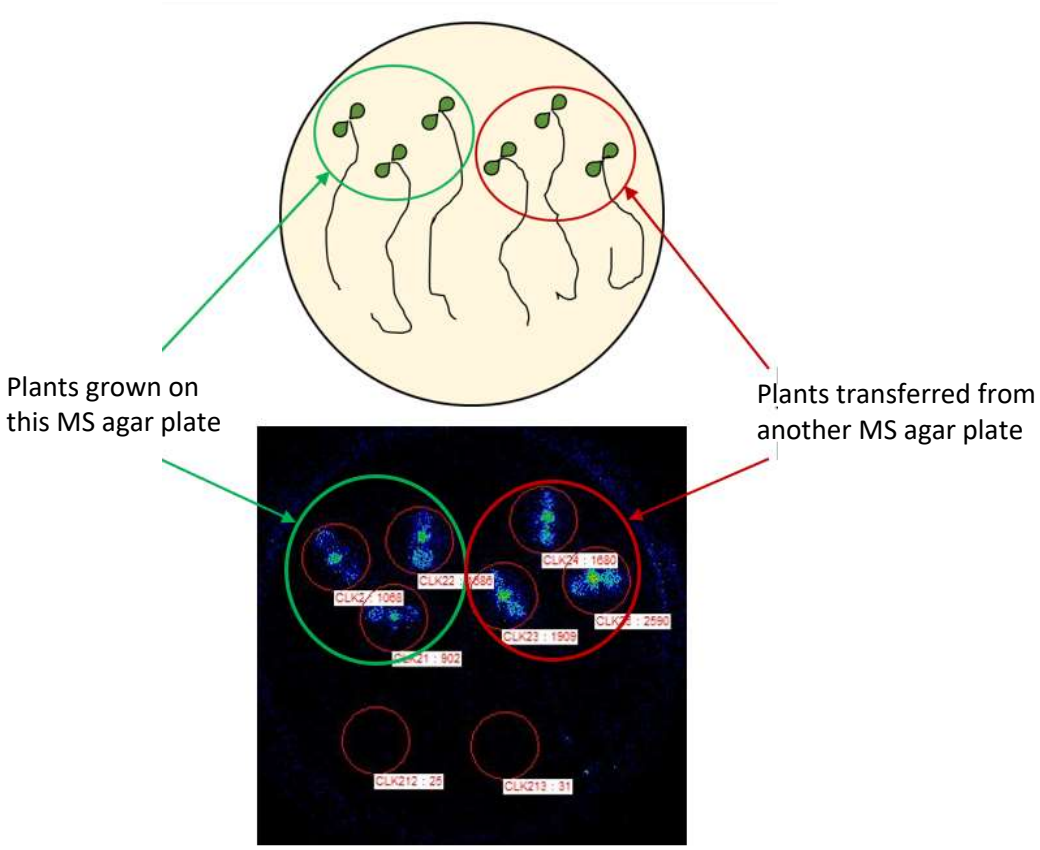

Figure 4.2. Arrangement of *CCA1::LUC* reporter plants used for testing effects of physical manipulation. Top image shows the arrangement of six *Arabidopsis* plants on a 60 mm MS plate: three remain on the original plate they were sown on, three were transferred from another plate. Bottom image shows bioluminescence output from these plants under the photon counting camera. Individually labelled circles represent the areas from which bioluminescence data was collected. The two circles at the base of the image were averaged to determine the background reading for photon counts.

The timecourse results are shown in Figure 4.3. Both sets of transgenic plants that had not been moved exhibited robust circadian rhythms. Luciferase bioluminescence from transgenic plants that has been transferred from a separate plate closely matched that of plants that were not moved. Analysing this data using FFT NLLS analysis (no detrending) gave the following period estimates (Figure 4.3b): *CCA1::LUC* unmoved 23.81 h ± 0.16, *CCA1::LUC* moved 23.92 h ± 0.07, *TOC1::LUC* unmoved 23.93 h ± 0.14, *TOC1::LUC* moved 23.56 h ± 0.30. *TOC1::LUC* plants that had been moved showed the most variability, with period estimates ranging from 23.07 h to 24.91 h. Although there were slight differences in period estimates, a one-way ANOVA revealed the differences between treatments were not statistically significant ($F = 0.845$, $df = 3$, $p > 0.05$).

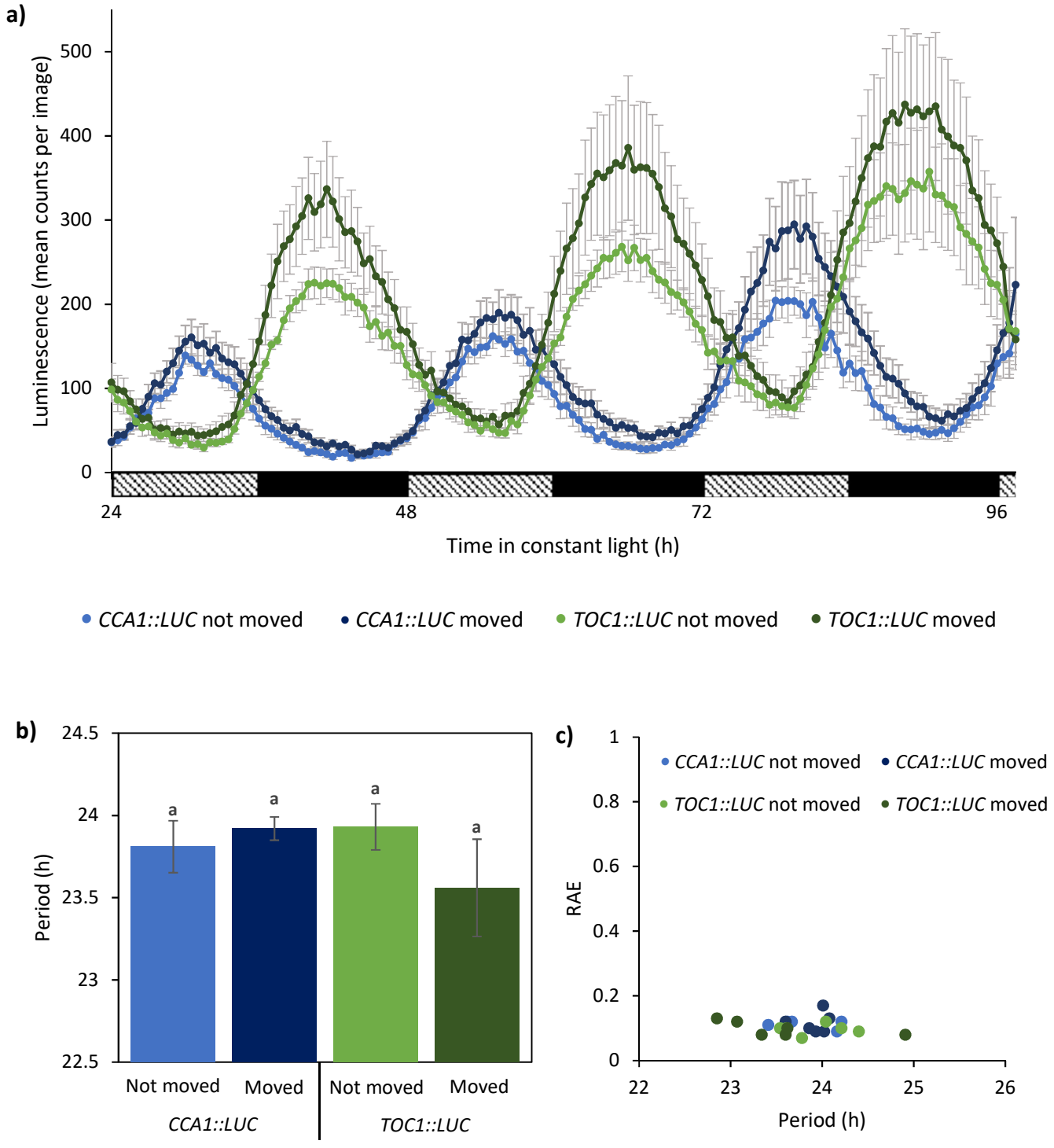

Figure 4.3. Moving plants does not affect *CCA1::LUC* or *TOC1::LUC* activity. a) Bioluminescence output from moved and unmoved transgenic shoots (n = 6). Hatched and solid bars represent subjective light and dark periods of the plant, respectively. b) Period estimates from circadian analysis using FFT NLLS analysis (without detrending). c) Comparison of period and relative amplitude error (RAE) for promoter-luciferase reporters. Error bars are representative of the standard error of the mean (SEM).

Phase estimates were as follows (Figure 4.4): *CCA1::LUC* unmoved 7.63 h ± 0.16, *CCA1::LUC* moved 7.89 h ± 0.41, *TOC1::LUC* unmoved 17.99 h ± 0.27, *TOC1::LUC* moved 18.86 h ± 0.71. The peak of both *CCA1* and *TOC1* promoter activity was consistent with the peak times documented in the literature (Wang & Tobin 1998; Strayer et al. 2000; Michael & McClung 2002; Noordally et al. 2013; Frank et al. 2018). A one-way ANOVA found differences between the 4 treatments ($F = 0.845$, $df = 3$, $p > 0.05$) but a post-hoc Tukey test demonstrated there were no significant differences in phase estimates between the 2 sets of *CCA1::LUC* plants ($p > 0.05$) and *TOC1::LUC* plants ($p > 0.05$). These results demonstrate that all transgenic plants exhibited robust circadian rhythms under constant light during the timecourse and that moving plants does not disrupt *CCA1* or *TOC1* promoter activity reported with bioluminescence imaging.

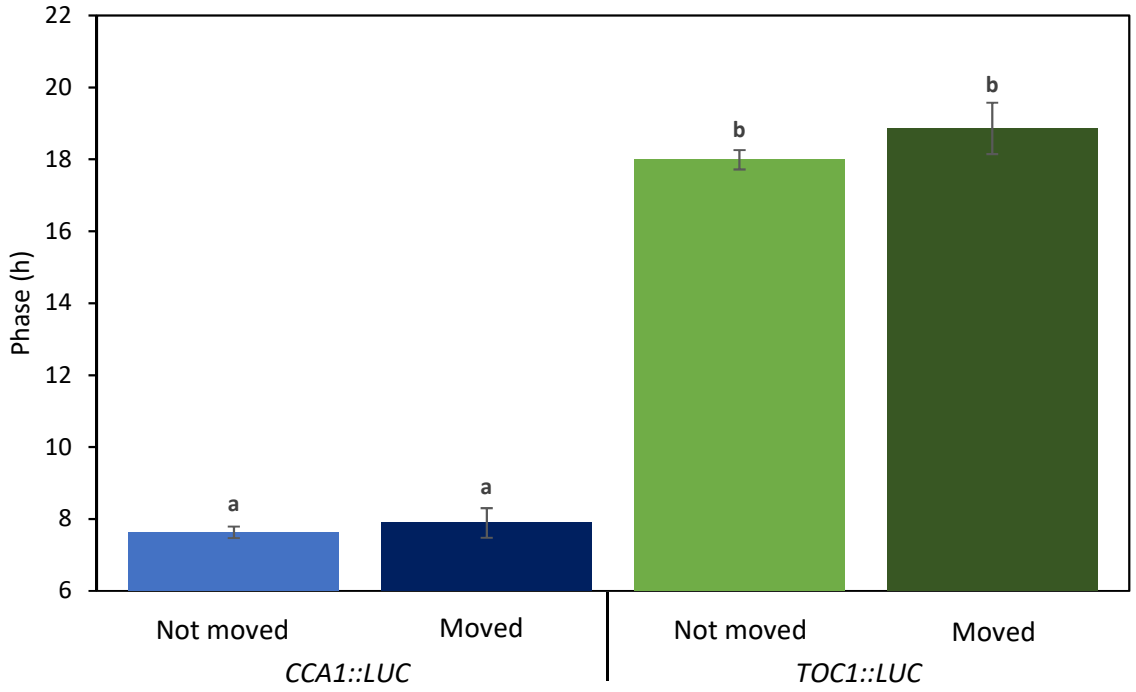

Figure 4.4. Moving plants does not affect the phase of circadian rhythms of *CCA1::LUC* and *TOC1::LUC* bioluminescence under constant light. Phase estimates from FFT NLLS analysis (n=6). Error bars are representative of the standard error of the mean (SEM).

4.2.2. Influence of inoculant media on plant circadian rhythms

It was also important to determine whether the LB media, used for the biofilm dip that inoculates the plant roots, might affect plant circadian rhythms. The LB media used was Miller's type. This contains a much higher salt content than other LB media: 10 g/L NaCl, compared to 5 g/L for Lennox LB or 0.5 g/L for Luria LB. It was therefore a concern that the high salt content may elicit an osmotic stress response in the plant and affect the oscillation of the core circadian genes, as has been

reported in other studies (Kumar et al. 2011; Habte et al. 2014). Habte and colleagues (2014) found osmotic stress at the barley roots caused a significant increase in expression of clock gene orthologues and increased the phase of expression of evening-clock genes in the shoot. A separate study by Kumar et al. (2011) observed downregulation of the rhythmic gene *WNK1* in rice.

To test for effects of the high salt LB on plant circadian rhythms, *CCA1::LUC* plants were grown in sterile plastic rings embedded in MS, with approximately 10 plants per well and 12 wells total (4 wells per treatment), as used in previous studies (Noordally et al. 2013; Belbin et al. 2017). At 16 d, different treatments were added to the plants in the wells: 1) untreated (control); 2) 50 µl LB added to wells or; 3) 50 µl of static culture of *B. subtilis* 168 grown in LB for 2 days added to wells. 15 min after plants were treated, 50 µl of 5 mM luciferin was added per well to provide the substrate for the bioluminescence reaction. Only 50 µl of luciferin was used in this experiment as the total well volume was 100 µl. These plants were placed under the ICCD camera and photon counts were measured from the transgenic plants every 30 min for 5 days with an integration time of 5 min and a 1% virtual neutral density filter. Between each measurement, plants were lit under constant red-blue light totalling 50 $\mu molm^{-2}s^{-1}$ (LL). Due to equipment constraints, only 1 plate could be analysed for bioluminescence signal at once so only *CCA1::LUC* plants were tested.

The effects of LB and *B. subtilis* application on *CCA1::LUC* activity are shown in Figure 4.5. Period estimates obtained using FFT NLLS analysis (no detrending) were as followed (Fig. 4.4b): 24.11 h ± 0.12 for untreated plants compared to 24.02 h ± 0.17 for plants treated with LB and 26.05 h ± 0.45 for plants treated with *B. subtilis* in LB. Although a one-way ANOVA demonstrated there were significant differences between these treatments ($F = 16.17$, $df = 2$, $p = 0.001$), a Tukey post-hoc test showed that LB had no effect on period respective to the untreated control ($p > 0.05$). Treating with *B. subtilis* in LB significantly increased *CCA1::LUC* period compared to the LB treatment ($p = 0.002$).

The peak of *CCA1::LUC* activity occurred slightly earlier with LB (Figure 4.6): 7.07 h ± 0.25 after subjective dawn for LB-treated and 6.2 h ± 0.35 after subjective dawn for untreated plants. Although there were significant differences in phase estimates between treatments ($F = 15.298$, $df = 2$, $p = 0.001$), the Tukey post-hoc test showed these significant differences were not between LB-treated and untreated plants. Whereas *B.* subtilis-treated plants had a significantly advanced phase, with *CCA1::LUC* activity peaking 3.99 ± 0.55 h after subjective dawn ($p = 0.001$).

Circadian FFT NLLS analysis for *B.* subtilis-treated plants yielded higher RAE values: mean RAE was 0.08 for LB treated plants compared to 0.22 for plants treated with *B. subtilis*. The slightly higher RAE values, combined with the reduced amplitude of *CCA1::LUC* oscillations (Fig. 4.5a), indicates that *CCA1::LUC* oscillations were possibly altered by treating the shoots with non-entrained *B. subtilis.*

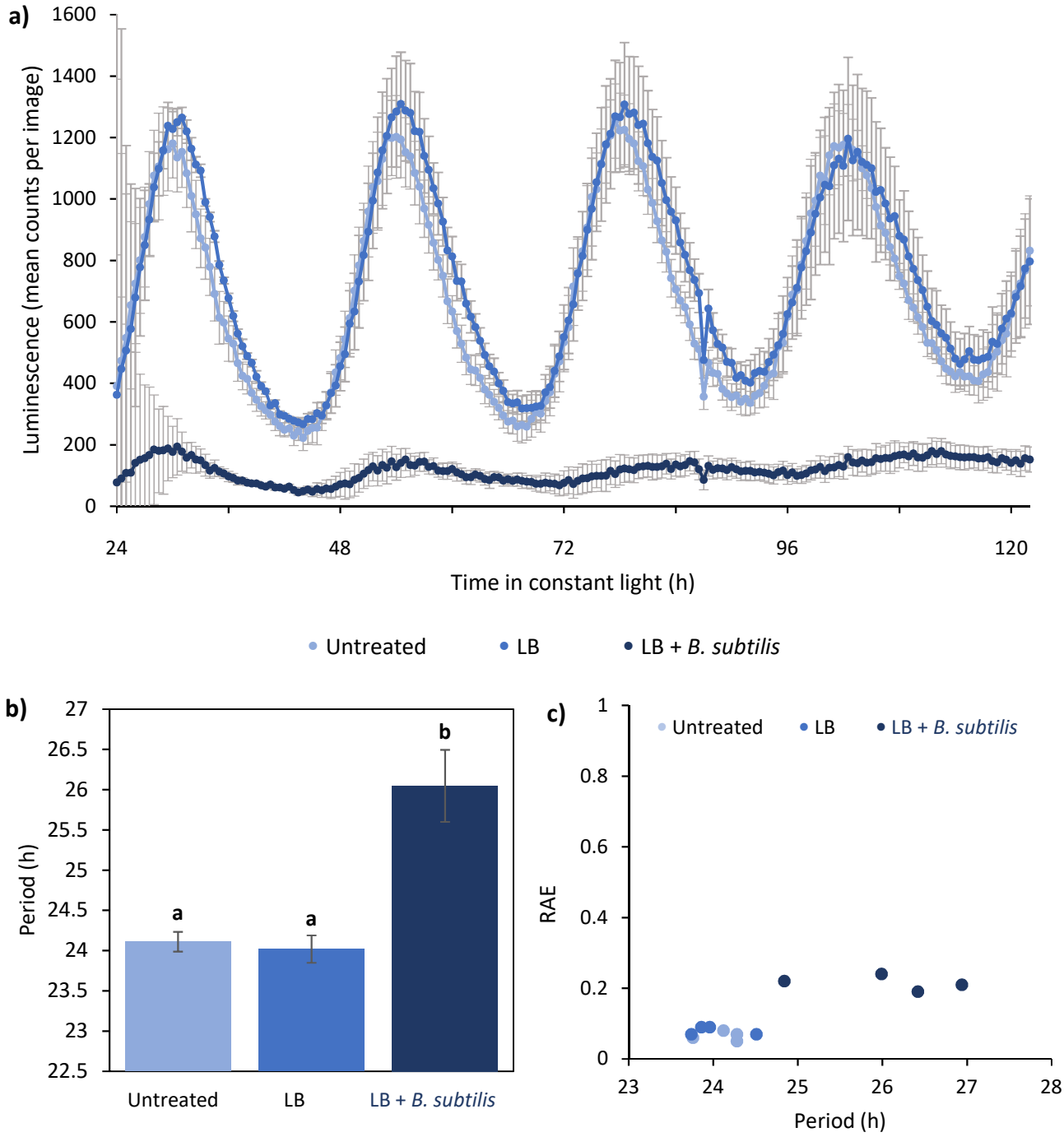

Figure 4.5. LB media does not affect *CCA1::LUC* activity under constant light. a) Bioluminescence output from untreated, LB-treated and *B. subtilis*-treated transgenic plants (n=4). Hatched and solid bars represent subjective light and dark periods of the plant, respectively. b) Period estimates from circadian analysis using FFT NLLS analysis (without detrending). c) Comparison of period and relative amplitude error (RAE) for promoter-luciferase reporters. Error bars are representative of the standard error of the mean (SEM).

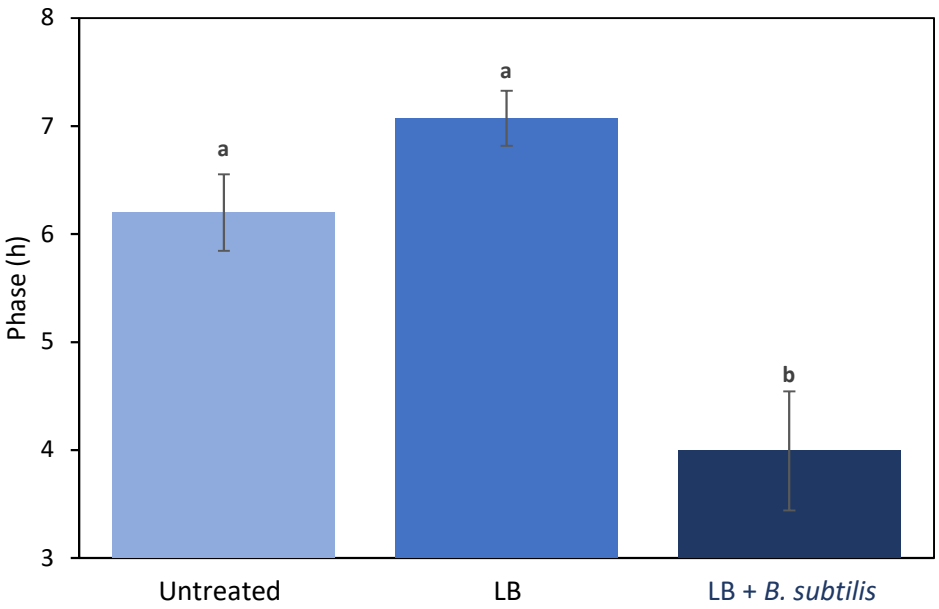

Figure 4.6. LB media does not affect the phase of circadian rhythms of *CCA1::LUC* bioluminescence under constant light. Phase estimates from FFT NLLS analysis (n=4). Error bars are representative of the standard error of the mean (SEM).

I reasoned that the *B. subtilis* inocula might have overloaded the plant shoots at such high bacterial densities (OD_{600} > 1.5), thus affecting the plant circadian rhythm and negatively affecting plant health. This negative impact on plant health is visible in Figure 4.7. The plants that were treated with *B. subtilis* appeared yellowed and had stunted growth compared to the untreated and LB-treated plants by the end of the timecourse. At this point it was decided to not use this well-based inoculation method for future experiments as plant health was adversely affected, instead only the roots should be inoculated.

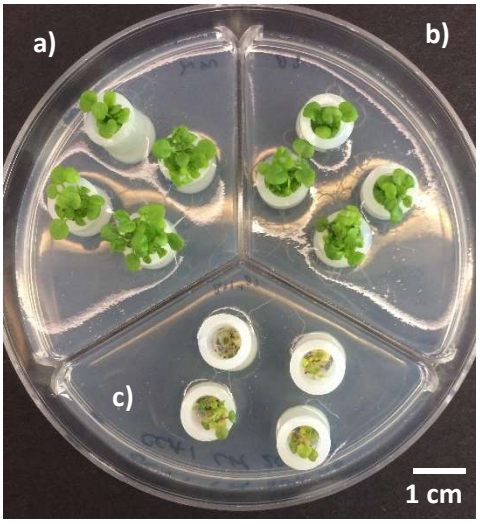

Figure 4.7. Condition of *CCA1::LUC* plants following different treatments: a) Untreated; b) LB-treated; c) *B. subtilis* treated. Image captured at the end of the 5-day timecourse when plants were 21 d. Approximately 10 plants per well. Stunted growth and chlorosis can be observed in *B. subtilis* treated plants.

4.2.3. Effects of *B. subtilis* root inoculation on *Arabidopsis* circadian oscillations

After establishing that moving the plants and treating with LB does not alter *CCA1* promoter activity, I evaluated the effects of different bacterial root inoculations upon *CCA1::LUC* activity. Firstly, the effect of a non-entrained bacterial root treatment was tested, followed by testing for effects of forward-entrained and reverse-entrained bacteria on *CCA1* promoter activity. The bacterial entrainment light regimes were as follows: no entrainment (culture at constant temperature and darkness), forward-entrainment (12 h:12 h light:dark cycles lit 9am to 9pm) or reverse-entrainment (12 h:12 h light:dark lit 9pm-9am). All plants were grown under the same light regime as forward-entrained bacteria. *B. subtilis* shoot inoculation in wells appeared to overload the plant and affect plant health, so the root biofilm dip method (see 3.2.3) was used for inoculating plants in these experiments.

4.2.3.1. Effects of non-entrained bacteria on *CCA1* promoter activity

To test for effects of *B. subtilis* without entrainment, *CCA1::LUC* plants were grown on vertical petri dishes until 14 d after germination and each treated with 100 µl of 10 mM luciferin in the evening. The following morning, roots were dipped in a non-entrained *B. subtilis* biofilm culture that had grown for 3 days at 37 °C and plants laid across half-strength MS in a 60 mm plate. 5 plants were

treated with the bacterial culture and placed on solid half-strength MS in one 60 mm plate, another 5 had their roots dipped in LB (the control) and placed in a separate plate. These plates were imaged immediately after inoculation using the photon-counting camera. Images were captured with a 5 min integration time and a 10% virtual neutral density filter every 30 min for 5 days.

Using FFT NLLS analysis (with linear detrending), the following period estimates were obtained (Figure 4.8): 24.28 h ± 0.19 for the LB-treated control plants compared to 23.39 h ± 0.22 for plants treated with non-entrained *B. subtilis*. Phase estimates were also altered for bacteria-treated plants (Figure 4.9). *CCA1::LUC* activity peaked 5.60 h ± 0.42 after subjective dawn for LB-treated and 6.94 h ± 0.72 after subjective dawn for *B. subtilis*-treated. RAE values were much lower for this experiment compared to *B. subtilis* shoot inoculations, suggesting robust circadian rhythms persist with *B. subtilis* root colonisation (Fig. 4.8c). Again, a one-way ANOVA test was used to test for differences between treatments and revealed *B. subtilis* significantly shortened the period of *CCA1::LUC* ($F = 9.156$, $df = 1$, $p = 0.016$) but did not significantly affect phase ($F = 2.593$, $df = 1$, $p > 0.05$).

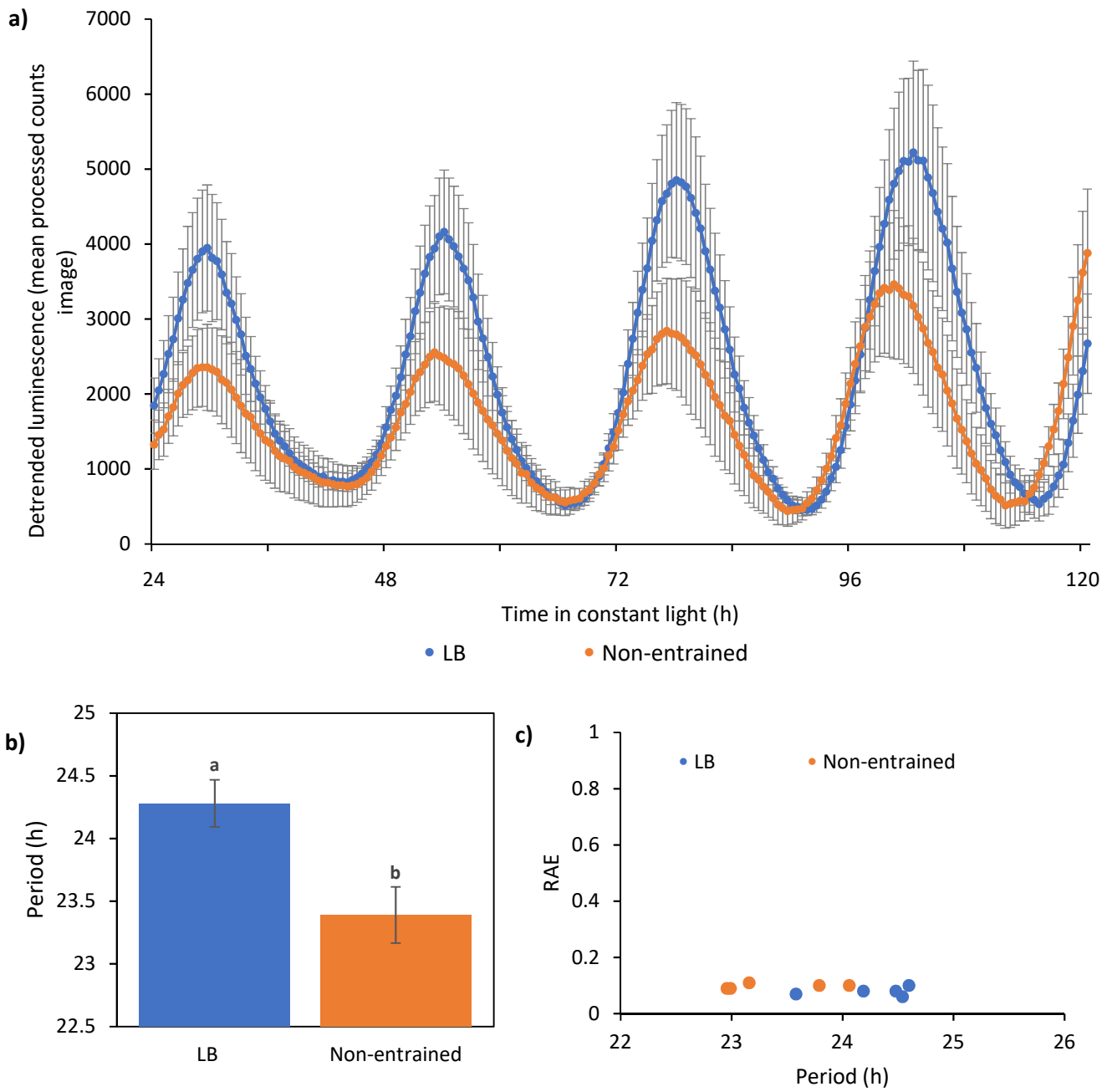

Figure 4.8. Bioluminescence output from *CCA1::LUC* plants treated with non-entrained *B. subtilis*. a) Timecourse data detrended using linear detrending (n=5). Hatched and solid bars represent subjective light and dark periods of the host plant, respectively. b) Period estimates from circadian analysis using FFT NLLS analysis showing clear period shortening with *B. subtilis* inoculation. c) Comparison of period and relative amplitude error (RAE) for promoter-luciferase reporters. Error bars are representative of the standard error of the mean (SEM).

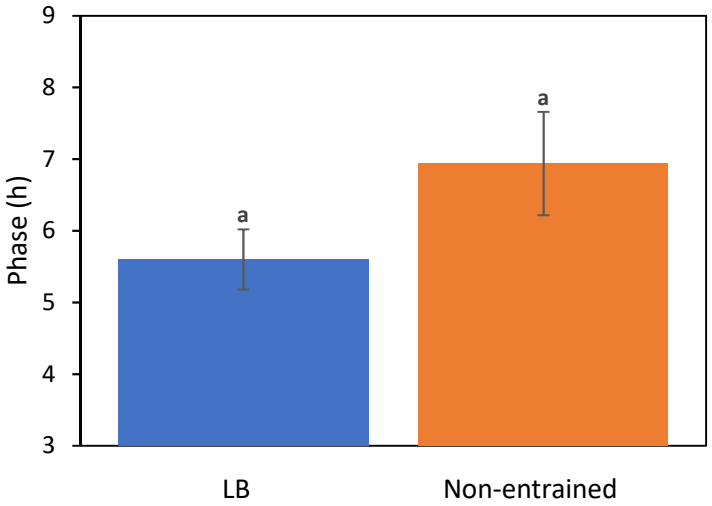

Figure 4.9. Phase estimates from *CCA1::LUC* plants treated with LB (control) or non-entrained *B. subtilis.* Estimates obtained from circadian analysis using FFT NLLS analysis with linear detrending (n=5). Error bars are representative of the standard error of the mean (SEM).

4.2.3.2. Effects of inoculating with forward-entrained bacteria on *CCA1* promoter activity

Next, I wanted to compare the effects of inoculating transgenic plants with forward-entrained bacteria versus non-entrained bacteria. This would later be followed by investigating the effects of inoculation with reverse-entrained bacteria. If bacteria with these opposing light regimes caused opposite changes to *CCA1::LUC* shoot oscillations, it might suggest that circadian entrainment of *B. subtilis* can subsequently alter circadian rhythms of the host plant. In addition, it would test whether these differential signals are travelling from the roots to alter plant circadian oscillations in the shoot, where *CCA1* promoter activity was being monitored.

First, an experiment was set up to test for the effects of forward-entrained *B. subtilis* on *CCA1::LUC* oscillations. Non-entrained cultures were grown in a 6-well plate wrapped with foil to prevent light entrainment. The root dip for the forward-entrained treatment was generated by growing bacteria in falcon tubes under the same lighting regime as the host plant (lit 9am to 9pm). In this experiment, both non-entrained and forward-entrained cultures were grown for 3 days in a plant growth chamber at 29 °C as was used in bacterial entrainment experiments by the Merrow lab (Zheng Chen 2018, pers.comm, 2 February). Forward-entrained cultures were grown on a shaking platform within the 29 °C plant growth chamber.

At 16 d, plants were treated with 100 µl 10 mM luciferin across the root and shoot in the evening. The following morning roots were treated with either: 1) LB; 2) non-entrained bacteria; or 3) forward-entrained bacteria. 4 plants were designated to each treatment and placed into separate

sections of a 3-section petri dish (Figure 4.10.). Plants were then immediately imaged under the photon-counting camera with images captured every 30 min for 5 min with a 10% virtual neutral density filter over the course of 5 days.

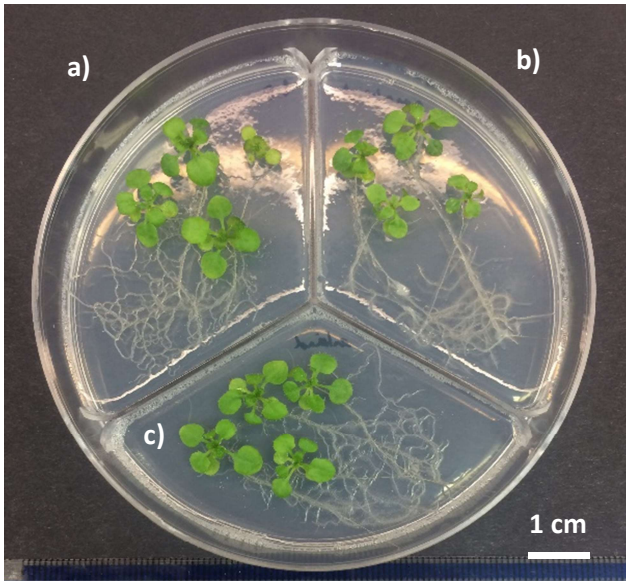

Figure 4.10. Transgenic *CCA1::LUC* plants following different root inoculations. 4 plants were designated per treatment: a) LB; b) Non-entrained bacterial root dip; c) Forward-entrained bacterial root dip. Inoculated plants placed on half-strength MS media in a 3-section 90 mm petri dish. Image captured at the end of the 5-day timecourse experiment.

Timecourse data were analysed using FFT NLLS analysis (with amplitude and baseline detrending) and gave the following period estimates (Figure 4.11): 23.49 h ± 0.11 for LB treated plants, 23.44 h ± 0.26 for plants treated with non-entrained *B. subtilis* and 23. 86 h ± 0.09 for plants treated with forward-entrained *B. subtilis.* Although there was a slight increase in period for plants treated with forward-entrained bacteria, this was not a significant difference respective to control plants or plants treated with non-entrained bacteria ($F = 1.527$, $df = 2$, $p > 0.05$). RAE values were low (≤ 0.12) for all plants treated with either bacterial treatment, suggesting *B.* subtilis root inoculations do not affect the robustness of plant circadian rhythms, unlike the RAE values reported for shoot inoculations in 4.2.2.

Phase estimates were as follows (Figure 4.12): 6.90 h ± 0.45, 7.11 h ± 0.14 and 7.34 h ± 0.11 for plants treated with LB, treated with non-entrained bacteria and treated with forward-entrained bacteria, respectively. A one-way ANOVA test showed these differences to not be significant ($F = 0.620$, $df = 2$, $p > 0.05$).

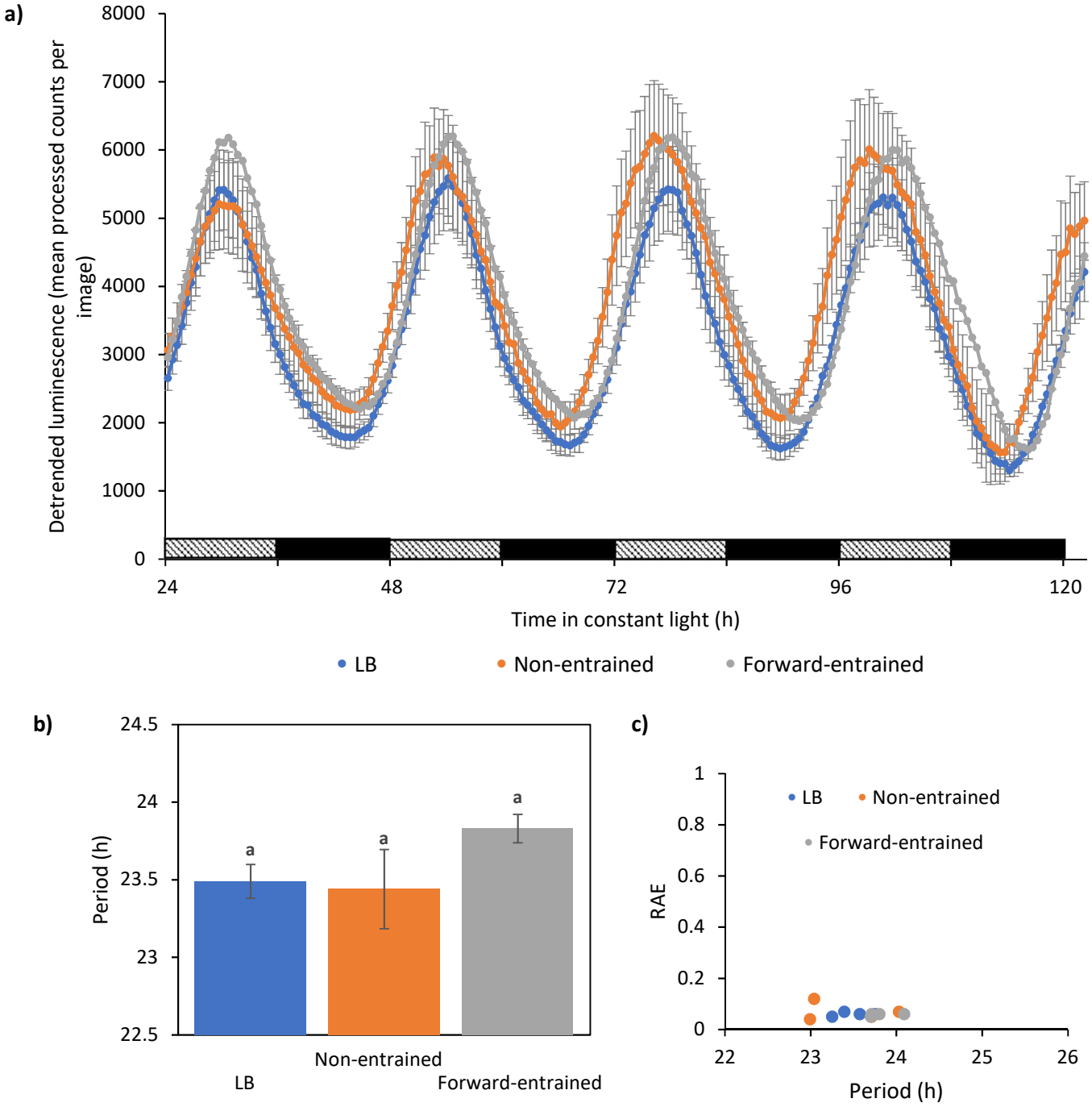

Figure 4.11. *CCA1::LUC* oscillations in transgenic plants treated with non-entrained *B. subtilis* or forward-entrained *B. subtilis*. a) Timecourse data detrended using amplitude and baseline detrending (n=4). Hatched and solid bars represent subjective light and dark periods of the host plant, respectively. b) Period estimates from circadian analysis using FFT NLLS analysis showing period lengthening with forward-entrained bacteria. c) Comparison of period and relative amplitude error (RAE) for promoter-luciferase reporters. Error bars are representative of the standard error of the mean (SEM).

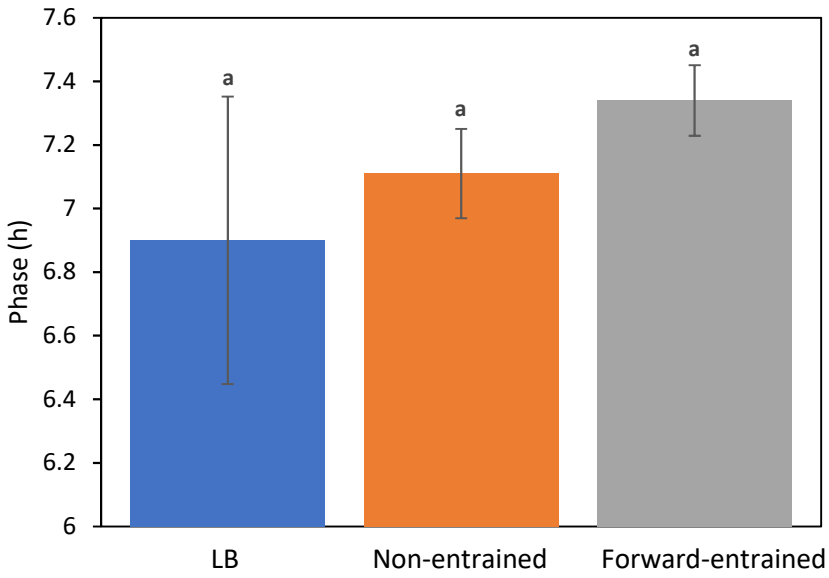

Figure 4.12. Phase estimates from *CCA1::LUC* plants treated with non-entrained *B. subtilis* or forward-entrained *B. subtilis.* Estimates obtained from circadian analysis using FFT NLLS analysis with amplitude and baseline detrending (n=4). Error bars are representative of the standard error of the mean (SEM).

4.2.3.3. Effects of inoculating with reverse-entrained bacteria on *CCA1* promoter activity

To test the effects of reversed-entrained bacteria on *CCA1* promoter activity, bacteria were grown under the opposite lighting conditions to their host plants. It was thought that a lengthening of *CCA1::LUC* period might be observed with reverse-entrained bacteria, since forward-entrained bacteria resulted in slight shortening of *CCA1::LUC* period. In this experiment, rather than growing the non-entrained bacterial treatment in static cultures, cultures were grown in falcon tubes wrapped in foil and placed on the shaking incubator in the plant growth cabinet with the reverse-entrained bacterial culture. Changing from static to shaking cultures was done to ensure both bacterial treatments were grown under the same conditions (excluding differences in lighting conditions). Shaking both cultures would prevent *B. subtilis* from carrying out anaerobic respiration as a result of lack of oxygen, which would alter gene expression and potentially affect the interaction between the host plant and bacteria (Nakano & Zuber 1998; Ye et al. 2000).

Plants were each treated with 100 µl 10 mM luciferin across the shoot and roots in the evening and then root-inoculated the following morning with one of 3 treatments: LB, non-entrained *B. subtilis* or reverse-entrained *B. subtilis*. Inoculated plants were placed on MS in a 3-section plate as in the previous experiment (Figure 4.10). Following root inoculations, images of the plant plate were captured every 30 min using the photon-counting camera with a 5 min integration time and a 10% virtual neutral density filter. In total, the experiment was repeated twice giving a total of 7 plants for

each root treatment. In the first replicate only 3 plants (instead of 4) were designated to each treatment, due to a lower availability of plants resulting from reduced germination success.

Circadian timecourse analysis using FFT NLLS analysis (with linear detrending) identified robust circadian rhythms in *CCA1* promoter activity (Figure 4.13). The following period estimates were obtained for *CCA1::LUC* oscillations in the 3 plant treatments (Figure 4.13b): 23.92 h ± 0.18 for LB-treated plants, 23.96 h ± 0.24 for plants treated with non-entrained *B. subtilis* and 23.59 h ± 0.16 for plants treated with reverse-entrained *B. subtilis.* Although administering reverse-entrained *B. subtilis* onto plant roots appeared to slightly shorten the circadian period of the host plant, a one-way ANOVA revealed this difference not to be significant ($F = 1.089$, $df = 2$, $p > 0.05$). Neither bacterial root treatments affected plant phase ($F = 0.060$, $df = 2$, $p > 0.05$; Figure 4.14): 6.76 h ± 0.38 for LB-treated, 6.98 h ± 0.69 for plants treated with non-entrained *B. subtilis* and 6.80 h ± 0.28 for plants treated with reverse-entrained bacteria.

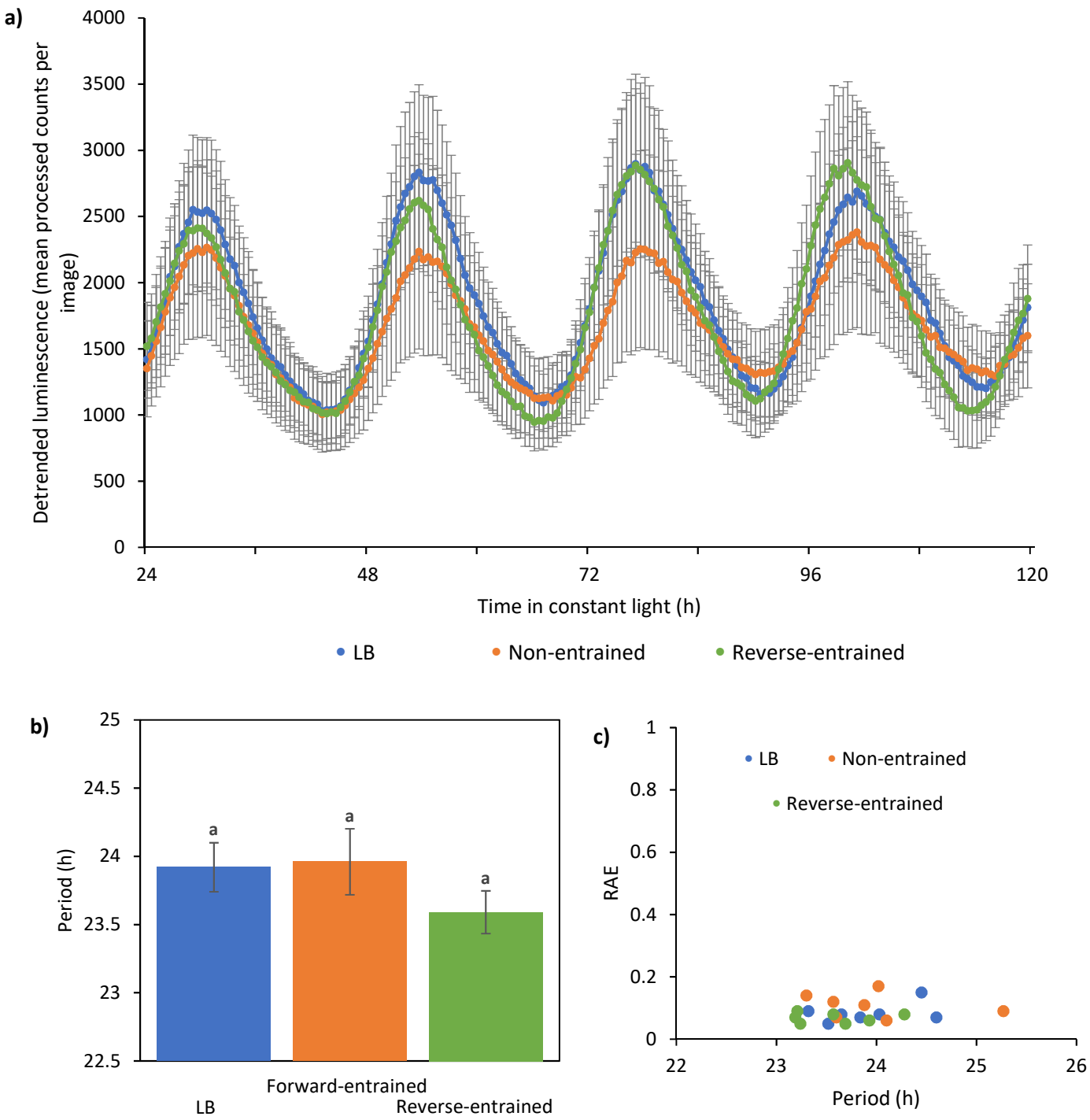

Figure 4.13. Circadian analysis of bioluminescence from *CCA1::LUC* plants treated with non-entrained *B. subtilis* or reverse-entrained *B. subtilis*. a) Timecourse data detrended using linear (n=7). Hatched and solid bars represent subjective light and dark periods of the host plant, respectively. b) Period estimates from circadian analysis using FFT NLLS analysis. c) Comparison of period and relative amplitude error (RAE) for promoter-luciferase reporters. Error bars are representative of the standard error of the mean (SEM).

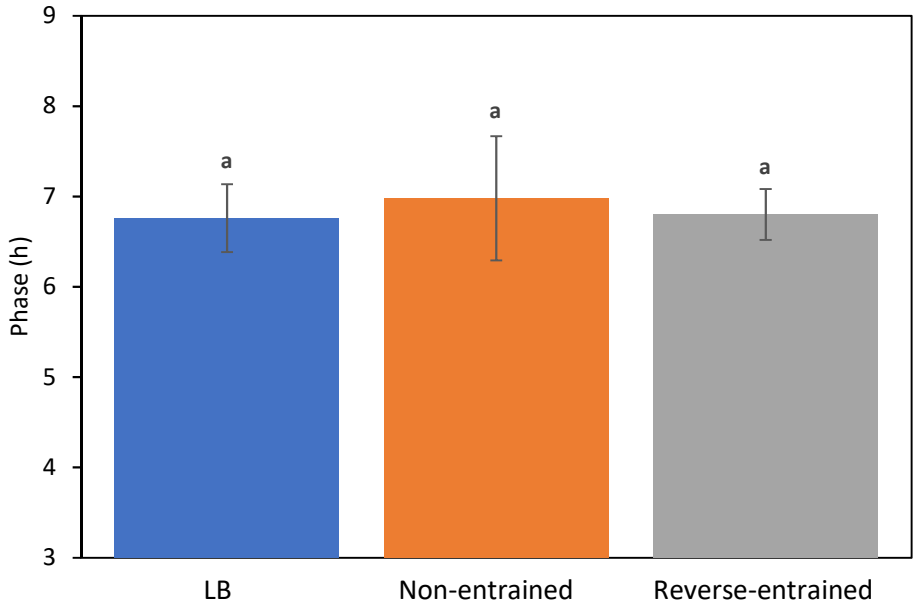

Figure 4.14. Phase estimates from *CCA1::LUC* plants treated with non-entrained *B. subtilis* or reverse-entrained *B. subtilis.* Estimates obtained from circadian analysis using FFT NLLS analysis with linear detrending (n=7). Error bars are representative of the standard error of the mean (SEM).

4.3. Discussion

Although the evidence for circadian rhythms in non-cyanobacterial prokaryotes remains very limited, the body of research is growing. This began with phylogenetic studies exploring *kai* gene homologues in non-photosynthetic bacteria by Dvornyk et al. (2003), and research uncovering circadian rhythmicity in gene expression in purple bacteria such as *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* (Van Praag et al. 2000; Min et al. 2005). More recently, evidence for the interaction of circadian clocks between organisms has been shown: the gut microbiota in mice and humans exhibits circadian rhythmicity which is affected by the host and in turn can affect gene expression in the host (Thaiss et al. 2014; Liang et al. 2015; Thaiss et al. 2016). Growth of *Arabidopsis* clock mutants has also been shown to alter the soil microbiome composition differently compared to soil surrounding wild-type plants (Hubbard et al. 2018a). However, research into the existence of circadian rhythms in the rhizosphere and their interaction with the plant circadian clock, remains largely unexplored.

The objective of this chapter was to investigate the effects of differentially entrained *B. subtilis* cultures on circadian rhythms in *A. thaliana* shoots. The Merrow group have obtained unpublished evidence that *ytvA* gene expression oscillates when entrained to light cycles (Zheng Chen 2018, pers.comm, 8 February), which provided the entrainment conditions used in this study. It was hypothesised that cultures of bacteria could be entrained to different light cycles and when in

association with plant roots, would alter the plant clock differently. This might be due to different temporal profiles of metabolite excretion from differentially-entrained bacteria. These patterns of metabolite excretion could provide entrainment cues for the plant via the roots. Secondary metabolites excreted from *B. subtilis* are known to alter the plant circadian clock. For example, growing plants on cytokinin-rich media is known to induce expression of the circadian clock genes *LHY* and *CCA1* but repress *TOC1* in a light-dependent manner, resulting in a phase-shift of the circadian clock (Zheng et al. 2006). Alternatively, secondary metabolites from *B. subtilis* may indirectly affect the plant circadian oscillator. For example, *B. subtilis* is known to alter ethylene content in the plant and ethylene is known to shorten plant circadian period (Xie et al. 2014; Haydon et al. 2017). Considering that *B. subtilis* dedicates approximately 4% of its genome to the production of secondary metabolites, it is possible that an unknown metabolite from *B. subtilis* may affect the plant circadian oscillator (Kunst et al. 1997).

The plant circadian clock is thought to be 'dynamically plastic' with several stimuli known to alter the phase and period of the clock, including ABA, sucrose, glucose, fructose, light intensity, high temperature, osmotic stress, iron and calcium irons (Somers et al. 1998; Hanano et al. 2006; Haydon et al. 2013; Salomé et al. 2013; Hansen et al. 2017; Ruiz et al. 2018; Webb et al. 2019). The magnitude of the response of the plant circadian clock to signals depends on the time of day. Therefore, if the same bacterial metabolite were excreted and taken up by the plant at different times during the day, it could elicit different responses by the circadian clock. If this bacteria-plant signalling pathway helped the plant match its circadian period closer to that of the environment, it would enhance plant fitness and reinforces the importance of the symbiotic relationship between rhizobacteria and plant hosts (Dodd et al. 2005). Considering that over 30% of the *Arabidopsis* genome is circadian-regulated, bacterial-induced changes to the plant circadian clock could have profound impacts on plant physiology and health (Bläsing et al. 2005; Michael et al. 2008).

In this study, changes to plant circadian clock were measured using transgenic circadian clock reporter plants, with *CCA1::LUC* plants used for the majority of experiments. Initial experiments in this chapter confirmed that the LB media and manipulation used in the developed inoculation method does not affect the circadian clock in *Arabidopsis*. This led to investigating the effects of different bacterial inoculations on the plant circadian clock. Inoculating plants with non-entrained *B. subtilis* gave different period estimates of *CCA1* promoter activity between experiments. When the culture was applied onto the shoots of plants grown in plastic wells, a lengthening of *CCA1::LUC* period was observed. However, this was likely due to the plant being overloaded by liquid culture and negatively affecting plant health, as was evident with reduced bioluminescence output from *CCA1::LUC* reporter plants accompanied by stunting and chlorosis of shoots. When non-entrained *B.*

subtilis was applied to roots, period shortening was observed. In later experiments alongside forward-entrained and reverse-entrained treatments however, the non-entrained treatment had no effect on plant period or phase. Inconsistencies in these results may be due to changing the conditions used for the generation of inocula. Initially, bacteria were grown for 3 days at 37 °C in constant darkness and applied to roots. However, this was changed to growth at 29 °C when the entrained bacterial treatments were used alongside. This change was done to replicate the entrainment conditions used in the Merrow lab. 37 °C is closer to the optimal growth temperature for *B. subtilis* and therefore bacterial cell densities would likely be higher at this temperature, although cell densities were not measured (Warth 1978). At higher cell densities, bacterial communities carry out quorum sensing whereby small signalling molecules called autoinducers alter bacterial gene expression in a cell density-dependent manner (Waters & Bassler 2005; Bareia et al. 2018). At least 89 genes are affected by quorum sensing in *B. subtilis.* Many of these genes are involved in the production of extracellular products, thus if the plant circadian clock is affected by bacterial metabolites, we would expect different responses at different bacterial cell densities (Comella & Grossman 2005). Additionally, if nutrients had become limiting in the culture grown at 37°C, cells may have undergone sporulation. The DNA-binding protein Spo0A is the master regulator for entry into sporulation and influences the expression of over 500 genes, resulting in cells differentiating into spores (Fawcett et al. 2000). These profound effects on global gene expression and cell state could reasonably affect the plant circadian clock differently to when the bacteria are in a biofilm community state. For future experiments, the optical density of bacterial cultures should be measured and normalised prior to use as inoculum to remove the confounding effects of bacterial cell density.

As hypothesised, treating roots with either forward-entrained or reverse-entrained *B. subtilis* gave opposing results. Forward-entrained bacteria slightly increased plant period, pulling the period closer to the 24 h cycle used for entrainment of both plants and bacteria. Reverse-entrained bacteria reduced the plant period, moving it away from 24 h. Although the changes in period length were opposite, a one-way ANOVA showed these differences were not significantly different. It should be noted that very small sample sizes (n=4) were used due to time and equipment constraints. The disparity between treatments may be significant with larger sample sizes so it would be valuable to use these preliminary data to perform a power calculation to determine the sample size that would be needed to confirm or reject these findings. It is also possible that *B. subtilis* may be affecting a plant circadian clock gene other than *CCA1,* so other plant clock genes should be considered. However, it may be that under these experimental conditions, entrained *B. subtilis* are not able to affect the circadian clock in *Arabidopsis.* Single-species experiments like this do not replicate the

complexity of the soil microbiome, the plant's natural substrate. The microbiome encompasses intra-species and inter-species interactions within a complex mixed substrate environment (Fierer 2017). This is a stark contrast to the monoculture of *B. subtilis* grown in simple LB media. It is therefore possible that circadian rhythms in non-photosynthetic bacteria like *B. subtilis*, would be better observed by using an intact rhizosphere community to replicate more natural conditions.

Another issue in these experiments is that *B. subtilis* rhythms were not confirmed before experimentation. Therefore, whether the bacterial cultures were successfully entrained to two opposing light cycles is uncertain. Nevertheless, the contrasting effects on *CCA1* period give an indication that the bacteria are sensing the two light cycles differently and using this signal to drive opposing output pathways which are sensed by the plant. In the future, rhythmicity in *B. subtilis* could be confirmed by measuring light output from the transgenic *ytvA::lux* strain (see Chapter 3) following different entrainment regimes. Alternatively, rhythmicity might be observed by growing the bacteria on LB agar plates supplemented with dye and measuring 'growth' rings on the plate over time, as done by Soriano et al. (2010). Since the experiments in this study were conducted, the Merrow lab have shown temperature cycles to act as stronger entrainment cues to *B. subtilis* than light (Zheng Chen 2018, pers.comm, 19 July). Therefore, growing the *ytvA::luxABCDE* strain under temperature cycles may result in stronger entrainment and more robust circadian rhythms.

Biofilm formation by *B. subtilis* on plant roots was also not examined in these experiments. It is therefore unknown how closely the bacteria were associated with the plant roots, a factor which may affect signalling between the host and bacteria. Looking ahead, the SYTO13 dye used for staining biofilms in chapter 3 would be a good candidate for confirming the plant-microbe interaction in future experiments (see section 2.5.2. for method).

To summarise, there is some promising evidence that *B. subtilis* entrained to contrasting light regimes can subsequently alter the period of *Arabidopsis* circadian clock, as shown by antagonistic changes to *CCA1::LUC* period. However, more experimental repeats may be required to validate these results. It is important to note that the conditions used in this study do not replicate the complex microbiome environment where these plant-microbe interactions naturally occur. It is possible that bacterial circadian properties are conditional and may be better observed in complex soil environments. Future experiments will require alterations to experimental methods such as: refining bacterial entrainment conditions (i.e. using temperature entrainment), confirming bacterial biofilm formation on roots and should also consider multi-species soil experiments to account for complexity of the microbiome. Nevertheless, these results provide an interesting avenue for future research.

Chapter 5: Discussion

Circadian rhythms are endogenous cycles of physiological processes that have a period of approximately 24 h. Circadian systems are well-characterised across the tree of life, including in plants, animals, fungi and cyanobacteria. These systems provide a fitness advantage by helping organisms coordinate responses with a changing external environment. (Ouyang et al. 1998; Sharma 2003; Woelfle et al. 2004; Dodd et al. 2005). Despite the detailed understanding that exists for circadian rhythms in many groups of organisms, the circadian biology of one of the most ubiquitous and economically important groups of organisms remains almost completely ignored: non-photosynthetic bacteria.

To date, the knowledge on bacterial circadian rhythms has mostly focused on cyanobacteria. The model cyanobacterium *Synechococcus elongatus* has a circadian system with a core oscillator composed of 3 proteins (KaiA, KaiB and KaiC) that operates through a phosphorylation-dephosphorylation system (Nishiwaki et al. 2007; Rust et al. 2007; Cohen & Golden 2015). It was initially thought that bacteria were unable to possess circadian rhythms since they divide multiple times in 24 h, a dogma known as the "circadian-infradian rule" (Ehret & Wille 1970; Edmunds 1983). However, it was later shown that the circadian clock in cyanobacteria functions well in cells dividing multiple times a day and operates independently from the cell division cycle (Mori et al. 1996; Kondo et al. 1997; Mori & Johnson 2001). Interestingly, cyanobacterial daughter cells inherit their circadian rhythm from the mother cell with little alteration to period or phase (Mihalcescu et al. 2004).

Evidence for circadian rhythms in non-photosynthetic bacteria has been found recently, including the presence of *kai* gene homologues in several bacteria and multiple studies showing diurnal variations in bacterial growth and gene transcription (Dvornyk et al. 2003; Min et al. 2005; Loza-Correa et al. 2010; Soriano et al. 2010; Paulose et al. 2016). These studies give some indication that non-photosynthetic bacteria may be capable of generating simple circadian rhythms, although whether these conform to the characteristics that classically define circadian rhythms such as temperature compensated and free-running under constant conditions, is yet to be shown (Pittendrigh 1960).

One important non-photosynthetic bacterium is *Bacillus subtilis*. This gram-positive bacterium can be isolated from a range of environments including several soil types. In fact, the *Bacillus* group is the most abundant genus of gram-positive soil bacteria, with up to 95% of soil sequences originating from *Bacillus* species (Garbeva et al. 2003). In the soil environment, *B. subtilis* regularly forms

associations with plant roots. This plant-microbe relationship is symbiotic: *B. subtilis* provides growth-enhancement and pathogen-protection for the plant, in return the host plant provides nutrients to support the growth of *B. subtilis* (Bais et al. 2004; Arkhipova et al. 2005; Ongena et al. 2005a; Swain & Ray 2006; Sharaf-Edin et al. 2008; Xie et al. 2014; Qiao et al. 2017). Like many microbes, *B. subtilis* also plays an important role in industry due to its production of secondary metabolites, including several antibiotics and enzymes (Palva 1982; Lesuisse et al. 1993; Olmos-Soto & Contreras-Flores 2003; Stein 2005). Investigating the presence of circadian systems in *B. subtilis* may improve our understanding of how these bacteria elicit growth-enhancing responses and pathogen-protection in host plants, as well as helping to increase yield of secondary metabolites in industrial settings.

Recently *B. subtilis* has shown potential for entrainment to light cycles and more recently, stronger entrainment to temperature cycles (Zheng Chen 2018, pers.comm, 8 February, 19 July). *B. subtilis* can sense light using the LOV domain-containing blue light-photoreceptor YtvA, making it reasonable to believe its use of light as a zeitgeber (Losi et al. 2002). Temperature sensing in *B. subtilis* is less studied, but the histadine kinase DesK is predicted to be involved in temperature sensing (Mansilla & de Mendoza 2005). These sensory capabilities, combined with its economic value and already well-studied genome and physiology, makes it a great candidate for early studies on bacterial circadian systems like this.

5.1. Summary: Influence of plant circadian rhythms on *B. subtilis ytvA::luxABCDE* oscillations.

I began this study by investigating the influence of plant circadian rhythms on *ytvA* promoter activity in transgenic *B. subtilis*. Initially, a significant amount of method development was required to formulate a protocol for the inoculation and imaging the *B. subtilis ytvA::luxABCDE* (abbreviated to *ytvA::lux*) strain on *Arabidopsis* roots. The method development section (see 3.2) demonstrated that inocula could be generated quickly by culturing the transgenic *B. subtilis* strain in liquid LB media. Chains of bacilli were observed when staining with crystal violet, demonstrating the strain's biofilm-forming abilities. The fluorescent dye SYTO13 revealed the close association of *B. subtilis* with *Arabidopsis* roots following inoculation. Following several root inoculation treatments and timecourse experiments, a biofilm root dip method was found to be the most effective for yielding consistently high bioluminescence output from the bacteria on the roots.

This method that was developed was subsequently used in timecourse experiments measuring bioluminescence output from *B. subtilis ytvA::lux* following inoculation onto oppositely-entrained host plants. Plants were grown in 24 h LD cycles, either lit 9am-9pm (forward-entrainment) or 9pm-

9am (reverse-entrainment). It was hypothesised that rhythms in *ytvA::lux* activity would oscillate with a period of 24 h but peak 12 h apart in the 2 sets of bacteria. This would identify that bacteria were entrained by the rhythms of the 2 sets of host plants, thus suggesting potential signalling of circadian timing information from the plant to bacteria.

Four repeats of this experiment were conducted, each yielding different timecourse profiles (see 3.3.2). Most interesting were the results from the first two timecourse experiments. Here, *ytvA::lux* activity oscillated with a period of 24 h for bacteria on both forward-entrained and reverse-entrained plants. Although there were differences in phase estimates between treatments, the peak of *ytvA::lux* activity was not 12 h apart between the 2 sets of bacteria, as was expected with oppositely-entrained plants. Phase and period estimates were also inconsistent between replicates. Inconsistencies in results between repeats were attributed to differences in several experimental factors: including differences in plant age, technical issues with temperature control and reduced biofilm formation in inocula used in some replicates (see Table 3.2).

Of particular interest, was the finding that circadian rhythms in *B. subtilis* may require biofilm-forming abilities. Biofilm formation is a requirement for successful root colonisation, so it is possible that biofilm formation is needed for the signalling of circadian timing information between plants and bacteria (Beauregard et al. 2013; Dietel et al. 2013). Free-living bacteria are markedly different to the biofilm state, comprising of a complex community with integrated signalling networks and multiple cell types (Vlamakis et al. 2008; Cairns et al. 2014). Perhaps, it is only in this complex biofilm community that external signals can be processed and used to generate circadian rhythms that persist through several bacterial generations. This conditional aspect of circadian rhythms has been documented in well-studied systems: many plant circadian clock genes that are rhythmic in constant light become arrhythmic in constant darkness (Millar et al. 1995b; Wang & Tobin 1998; Covington et al. 2001; Más et al. 2003b). In addition, at cold temperatures circadian rhythms are damped in many organisms (Zimmerman 1969; Bieniawska et al. 2008; Murayama et al. 2017). Circadian rhythms in *B. subtilis* may be conditional on an element of biofilm formation on plant roots.

Future experiments should ensure plant age, lighting conditions and temperature are kept constant to help avoid inconsistencies between experiments. In addition, the SYTO13 fluorescent dye should be used alongside each timecourse experiment to determine the extent of bacterial biofilm formation on the roots. This will help identify whether *B. subtilis* biofilm formation is required for bacterial circadian rhythms that are influenced by the host circadian rhythm.

5.2. Summary: Impacts of root colonisation by *B. subtilis* on circadian rhythms in *A. thaliana* shoots.

B. subtilis has been shown to have a profound effect on plant gene expression, following root colonization (Ongena et al. 2005b). These effects on plant gene expression have focused on the upregulation of plant defence genes (Nakkeeran et al. 2006; Gond et al. 2015; Fousia et al. 2015). I wanted to examine how the bacteria may affect the expression of plant circadian clock genes.

Rhythmic secretion of bacterial secondary metabolites may provide different effects on the plant circadian clock. The period and phase of the plant circadian clock are proposed to be 'dynamically plastic', changing in response to several stimuli (Somers et al. 1998; Hanano et al. 2006; Haydon et al. 2013; Salomé et al. 2013; Hansen et al. 2017; Ruiz et al. 2018; Webb et al. 2019). Interestingly, the bacterial flagellar peptide flg22 from *Pseudomonas syringe* has been shown to significantly shorten *CCA1* period (Zhang et al. 2013). The plant response to bacteria-derived metabolites may also be dependent on when they are received by the plant, through a process known as circadian gating. With 4% of the *B. subtilis* genome involved in the production of secondary metabolites, it is reasonable to believe that another bacterial product could affect the expression of plant circadian clock genes or effect plant health, depending on the time of day (Kunst et al. 1997).

Using two opposing light regimes (termed forward-entrainment and reverse-entrainment), non-transgenic *B. subtilis* cultures were entrained to two opposing cycles (or given no entrainment) and inoculated onto plant roots. It was expected that the differentially-entrained bacteria would affect the period or phase plant circadian clock in opposing ways. Transgenic *CCA1::LUC* reporter plants were used to measure changes over time to the period and phase of the circadian rhythms in the activity of this promoter. Initial control experiments found the manipulation and bacterial growth media used in the developed method did not disrupt the plant circadian rhythm (see Fig. 4.3 and 4.4). Inoculating *Arabidopsis* roots with *B. subtilis* grown without entrainment gave mixed result: both period lengthening, period shortening and no effects on *CCA1::LUC* period were observed (see Figs. 4.5-4.14). These differences could be the result of changes to inoculation techniques (well-based shoot inoculations to root inoculations) and bacterial culture conditions (37 °C to 29 °C, static to shaking). Changing culture conditions may have affected bacterial cell density, cell signalling and subsequently bacterial gene expression and metabolite production. This would likely affect the plant circadian clock differently.

B. subtilis entrained to opposite lighting regimes had opposite effects on *CCA1::LUC* period: forward-entrained bacteria slightly increased clock period (pulling it closer to the 24 h entrainment cycle) and reverse-entrained bacteria slightly decreased clock period (see Fig. 4.11 and 4.13). This agrees with

the idea that the period of the plant clock is 'dynamically plastic' (Webb et al. 2019). However, these period differences were not statistically significant. Considering very small sample sizes were used for most experiments, it would be valuable to use these preliminary data to perform a power calculation to determine the sample size that would be needed to robustly confirm or refute this hypothesis. It should also be considered that *B. subtilis* may be affecting plant circadian clock genes other than *CCA1*, so other clock gene should be tested.

One key point arising is the possibility that circadian rhythms in *B. subtilis* are conditional and only occur when the bacteria are in the natural microbiome environment with intra- and inter-species interactions. Testing bacterial circadian rhythms in the future may require the use of complex soil microbiomes instead of single-species experiments.

5.3. The advantage of circadian rhythms in plant-bacterial associations

A symbiotic relationship involving signalling of circadian timing information between plants and rhizobacteria is analogous to that of the gut microbiome and host. The gut microbiota is affected by the host: abundance of bacteria in the mice gut exhibits circadian rhythmicity which is dependent on the timing of food intake and host clock functioning (Liang et al. 2015). In return, circadian rhythms in gut microbiota affects host circadian clock function. Interestingly, rhythmic host physiology is altered beyond the intestines, with changes to transcriptional oscillations in the liver (Leone et al. 2015; Murakami et al. 2016; Thaiss et al. 2016). Disruptions to the host circadian rhythm and subsequently the composition of the microbiome affects host health, increasing disease susceptibility (Thaiss et al. 2014; Thaiss et al. 2016). This bidirectional signalling confers a fitness advantage for the mammalian host and generates circadian rhythms in the microbiota which may also affect bacterial fitness.

The effect of crosstalk between plants and soil bacteria on plant circadian clock function has also been demonstrated. Zhang et al. (2013) showed infection with *Pseudomonas syringae* significantly shortened *Arabidopsis CCA1* period. In another study, growing plants in disrupted (autoclaved or filter sterilised) microbiomes lengthened the circadian period by 1 h relative to plants grown in intact microbiomes (Hubbard et al. 2018b). The same study also found a microbiome altered by a previous plant could pass this information on to other hosts: for example, the period of *ztl-1* long period mutants was significantly shortened when grown on soil previously occupied by *toc1-21* short period mutants. Plant circadian clock functioning appears to in turn affect the soil microbiome. Rhizosphere community structure differs between day and night and is significantly altered with circadian clock mutant plants. Again, information from previous plants was held in the microbiome: wild-type plants

germinated later and were significantly smaller when grown in soils previously occupied with clock-mutant plants (Hubbard et al. 2018a).

Rhizobacterial effects on the plant circadian clock may confer a fitness advantage. For plants, one benefit could be the ability to use temporal cues from root-colonising bacteria to anticipate the secretion of bacterial secondary metabolites. Therefore, proteins associated with the uptake and metabolism of these metabolites can be synthesised at the correct time. Root transport proteins to consider include the nitrate uptake transporters such as NITRATE TRANSPORTER 2 (NRT2) transporters since nitrate uptake exhibits diurnal variation and phosphate transporters such as PHT4;1 which is circadian clock-regulated (Ohyama et al. 1989; Orsel et al. 2002; Shin et al. 2004; Keltjens & Nijënstein 2008; Wang et al. 2011). However, circadian profiles of metabolite secretion from bacteria have not yet been reported so the exact plant transport proteins involved in this interaction are undetermined.

Alternatively, temporally secreted metabolites from bacteria may be used alongside other zeitgebers such as light and temperature to help reinforce the plant circadian rhythm to better match it to the environment. A plant circadian period that better matches that of the environment results in plants fixing more carbon, containing more chlorophyll, having a higher vegetative biomass and surviving better (Dodd et al. 2005). A potential reinforcement of plant circadian rhythms was observed in this study, with forward-entrained bacteria bringing the plant circadian period closer to the 24 h entrainment cycle (see Fig. 4.11).

For bacteria, possessing a circadian system that can be entrained by signals from plant roots could also provide several benefits. Some plant exudates, including citrate, flavonoids and mugineic acid-family phytosiderophores (MAs), have diurnal patterns in their secretion (Hughes et al. 1999; Watt & Evans 1999; Nagasaka et al. 2009; Badri et al. 2010). Anticipating the secretion of plant-derived metabolites may allow soil bacteria to synthesise proteins involved in their uptake and metabolism just prior to their secretion from plant roots. One protein that may be of interest in *B. subtilis* include the citrate transporter CitM (Warner & Lolkema 2002). Anticipating plant metabolites would avoid the synthesis of these proteins occurring at a time when the metabolites are not being secreted from the plant and would provide a competitive advantage against other soil bacteria for nutrients. Increasing growth by beneficial rhizobacteria could in turn provide more protective and growth-enhancing benefits to the host plant.

5.4. Broader relevance of these findings

The results for potential circadian rhythmicity in *B. subtilis* build on the evidence for a possible entrainable circadian system in non-photosynthetic bacteria. This may have implications for use of bacteria in industry. The production of secondary metabolites such as enzymes and antibiotics makes *B. subtilis* an important contributor to many industries. Targeting the rhythmic behaviour of this bacterium, such as by directing nutrients to certain times of day, could increase both the quantity and quality of bacterial products. In medicine, administrating antibiotics at specific times of day or targeting bacterial circadian gene pathways could slow the development of disease.

The finding that *B. subtilis* may affect the plant circadian clock could be of importance for its use in agriculture. As discussed, *B. subtilis* is known to have a significant impact on plant gene expression (Ongena et al. 2005b; Hubbard et al. 2018b). Volatile organic compounds (VOCs) released from *B. subtilis* alter expression of over 600 genes in *Arabidopsis* related to metabolism, auxin synthesis, cell wall modification, chloroplast function, stress responses and signalling (Zhang et al. 2007). With over 30% of the *Arabidopsis* genome under circadian control, the changes to plant period observed in this study will likely have major impacts on plant physiology and health (Bläsing et al. 2005; Michael et al. 2008). By better understanding how soil bacteria affect the plant circadian clock, we may be able to alter the functioning of bacterial circadian clocks to enhance plant productivity.

5.5. Future work

5.5.1. Circadian clock gene homologues in bacteria

This study has illustrated the need to improve and standardise experimental procedures, such as ensuring examination of plant-bacterial associations using microscopy and refining bacterial entrainment regimes. However, there are other interesting avenues for future studies on bacterial circadian rhythms.

Exploring the presence of circadian gene homologues and circadian clock-associated protein domains in non-cyanobacterial prokaryotes provides a logical step for exploring novel circadian systems. This project explored the latter: bacterial genes encoding proteins with PAS domains, as present in many circadian clock components (Antoch et al. 1997; Crosthwaite et al. 1997; Nelson et al. 2000; Somers et al. 2000; Schultz et al. 2001; Hennig et al. 2009). Future work could look at cyanobacterial *kai* gene homologues in non-photosynthetic bacteria. I carried out tBLASTn searches using Kai amino acid sequences from the model cyanobacteria *Synechococcus elongatus* and found highly similar homologues in several non-cyanobacterial prokaryotes. The KaiC protein produced hits with high similarity in soil bacteria including nitrogen-fixing *Bradyrhizobium* sp. ORS278 (46.45%

sequence identity), the soil bacterium *Gemmatimonadetes kalamazoonensis* (46.12% sequence identity) and the root-nodulating *Mesorhizobium opportunistum* (46.38% sequence identity) (Giraud et al. 2007; Reeve et al. 2013; DeBruyn et al. 2014). KaiB also produced a hit with *Bradyrhizobium* sp. ORS278 (56.47% sequence identity) among others (Giraud et al. 2007). However, no hits were found for KaiA in non-cyanobacterial prokaryotes. This is in agreement with the studies by Dvornyk et al. (2003) and Loza-Correa et al. (2010) who found *kaiA* is only present in cyanobacteria. *B. subtilis* did not produce hits with any of the cyanobacterial Kai proteins, so any circadian system is likely act through a different set of genes.

Generating transgenic bacteria with reporters for *kaiB* and *kaiC* gene homologues and measuring rhythmic promoter activity could give an insight into whether these bacteria can use a Kai-based circadian system without *kaiA*. Interestingly, Ma et al. (2016) found that although daily rhythms in nitrogen fixation the purple bacterium *Rhodopseudomonas palustris* (harbouring *kaiB* and *kaiC* homologs only) exhibited rhythmicity that were somewhat temperature compensated, these rhythms did not persist in free-running conditions. This study also found *kaiC*-dependent growth enhancement under LD cycles but not under constant conditions, suggesting an adaptive value of *kaiC* even in the absence of *kaiA*. The authors termed this circadian clock a "proto-circadian oscillator" with some but not all the classically-defined characteristics of circadian clocks. These findings question the requirement to use a different set of characteristics for defining circadian rhythms in non-cyanobacterial prokaryotes.

5.5.2. Unpicking entrainment signals

As discussed, there is a need to refine the light and temperature regimes used for entraining *B. subtilis*. It would also be valuable to study plant and bacterial metabolites that may act as entrainment cues or influence plant and bacterial circadian rhythms in this interaction.

Multiple papers have already reported on diel variation in the secretion of several root exudates, giving potential candidates for metabolites influencing bacterial circadian rhythms (Hughes et al. 1999; Watt & Evans 1999; Nagasaka et al. 2009; Badri et al. 2010). However, to date no studies have been conducted on rhythmicity of secondary metabolite excretion from bacteria. Analysing the temporal profile of metabolite excretion from *B. subtilis* may provide insights into signals that act as inputs into the plant circadian clock. This information could be used to design root treatments that affect the plant circadian clock and plant physiology when applied at certain times of day.

5.6. Conclusions

This study provides the first evidence for a bidirectional interaction of circadian rhythms between plants and bacteria, building on previous work indicating effects of the microbiome on plant clock function (Hubbard et al. 2018a, 2018b). I obtained some evidence suggesting that circadian rhythms in *Arabidopsis* generate circadian oscillations in *B. subtilis ytvA* promoter activity and that *B. subtilis* cultures entrained to opposing light-regimes might have opposite effects on the period of *CCA1* promoter activity. However, further repeats may be required to determine the validity of these findings. Future work should seek to pinpoint the signals responsible for bacterial and plant entrainment in this relationship and the bacterial genes involved in their potential circadian clocks. Considering the use of intact soil microbiomes as opposed to single species experiments may also be important for future experiments, as bacterial circadian rhythms may be conditional on an element of complex bacterial communities. The potential for circadian rhythms in non-photosynthetic bacteria has implications for both their use as protein secretion factories in industry and for the treatment of microbe-associated diseases. In agriculture, bacterial-induced changes to plant circadian rhythms may have profound impacts on plant physiology and health.

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Appendices

Stain	Components	Principle	Excitation/ emission	Price	References
FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain	N/A – ready to use	Labels most classes of proteins, including glycoproteins, phosphoproteins, lipoproteins, calcium binding proteins, fibrillar proteins Stain EPS of biofilm May stain plant cell wall.	280, 450/610 nm	£87.75 for 200 ml	No literature for staining *Bacillus* or staining plant roots.
LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit	Two-colour fluorescence assay - SYTO® 9 green-fluorescent nucleic acid stain -Red-fluorescent nucleic acid stain, propidium iodide.	SYTO9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. But propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Also stains plant nuclei.	480/500 nm for SYTO 9 stain 490/635 nm for propidium iodide	£399 for 1 kit	See image in Bais et al. (2004): *B. subtilis* biofilm on *Arabidopsis* plant roots (the SYTO9 component)
SYTO® 9 green fluorescent nucleic acid stain	5 mM solution of SYTO9 in DMSO (but dilute for use)	Cell-permeant nucleic acid stains that show a large fluorescence enhancement upon binding nucleic acids (RNA and DNA) Stains bacteria and plant nuclei.	485/498nm	£230 for 100 µl	Banet et al. (2014) stained biofilm formation on coverslips
SYTO™ 13 Green Fluorescent Nucleic Acid Stain	5 mM solution of SYTO13 in DMSO (but dilute for use)	Binds to nucleic acids Also stains bacteria and plant nuclei.	488/509nm	£213 for 250 µl	Rudrappa et al. (2008) stained biofilms of *B. subtilis* FB17 strain on *Arabidopsis* roots

Table A1. Review of fluorescent dyes suitable for staining *B. subtilis* biofilm formation on *Arabidopsis* roots.