Melatonin Impacts Soil Microbes and Microbial Community Structures in Agricultural Soils under Abiotic Stress

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List of Abbreviations

APX	Ascorbate peroxidase
ARISA	Automated Ribosomal Intergenic Spacer Analysis
ASMT	*N*-acetylserotonin methyltransferase
CAT	Catalase
Cd	Cadmium
CIOMT	Caffeic acid *O*-methyltransferase
DDC	5-hydroxy-L-tryptophan decarboxylase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EtOH	Ethanol
Expt	Experiment
FW	Fresh Weight
GRT	Glutathione reductase
IAA	Indole-3-acetic acid
MT	Melatonin
OTU	Operational Taxonomic Unit
PAA1	Polyamine *N*-acetyltransferase
PCR	Polymerase Chain Reaction
POD	Peroxidase
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
SNAT	Serotonin *N*-acetyltransferase
SOD	Superoxide dismutase
T5H	Tryptamine 5-hydroxylase
TDC	Tryptophan decarboxylase
TPH	L-tryptophan hydroxylase

Abstract

Melatonin is a secondary metabolite produced ubiquitously in nature. Recent evidence indicates that melatonin enhances plant tolerance to abiotic and biotic stresses, leading to speculation of future applications of melatonin in agriculture. There is little knowledge relating to responses and potential roles of melatonin in soil microbes. Aims of this project were to: 1) assess (using automated ribosomal intergenic spacer analysis) whether exogenous melatonin affected microbial community responses in three agricultural soils under abiotic stress [cadmium or salt]. ; 2) determine bacterial taxa responding to melatonin in two agricultural soils using next generation sequencing; 3) assess *in vitro* growth responses of three soil-borne fungal phytopathogens (*Sclerotinia sclerotiorum*; *Botrytis cinerea* and *Fusarium oxysporum* f.sp. *vasinfectum*) to melatonin (20 – 4000 µM) under two abiotic stresses (2.5% v/v ethanol and cold (4^{o}C)); 4) explore if the immediate availability of exogenous melatonin (200 µM) at the site of infection altered the disease development of *S. sclerotiorum* on *Nicotiana tabacum* leaves.

The results of these investigations showed that agricultural soil microbial communities were impacted by melatonin under abiotic stresses. Some plant growth promoting rhizobacteria showed significant responses to exogenous melatonin. Melatonin enhanced *in vitro* radial growth of all three fungi under abiotic stress conditions, while the infection capability of *S. sclerotiorum* on tobacco (*N.tabacum*) plants was unaffected by melatonin . The findings provide valuable insights into the interaction of soil microbes with melatonin. This study is the first to report the effects of melatonin on agricultural soil microbial communities both under abiotic stress and unstressed conditions. This is also the first study to demonstrate that exogenous melatonin enhances tolerance to abiotic stresses for pathogenic filamentous fungi. Understanding how soil microbes are affected by melatonin may provide vital information regarding the utility of melatonin in future agricultural practices.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

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Signed:

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Aho Mitakuye Oyasin

Chapter 1 General Introduction

1.1 Overview

Global crop production is being greatly impacted by the increasing exposure to various abiotic stresses caused by natural events and anthropogenic activities. Over the past decades, climate change has had a direct impact on crop production for agricultural practices across the world (Ali *et al.*, 2017; Leng, 2017; Matiu *et al.*, 2017). Increasing temperatures due to global warming have resulted in more frequent occurrences of extreme climatic conditions, including droughts and heat waves (Van Gorsel *et al.*, 2016). These abiotic stresses heavily impact crop yield directly by altering plant cellular activities, as well as indirectly by affecting soil health (Wang *et al.*, 2003; Liu *et al.*, 2014; Lu *et al.*, 2014; Suszek-Łopatka *et al.*, 2019).

Soil salinity is an example of a major abiotic stressor in agricultural practices throughout the world (Zhu, 2001), with up to 2 million hectares of agricultural land depleted each year worldwide due to soil salinization (Bencherif *et al.*, 2015; Ke *et al.*, 2017). Soil contamination due to anthropogenic activities such as mining, agricultural practices and pollution can also reduce crop productivity by introducing harmful chemicals into the environment (Barletta *et al.*, 2019; Evans *et al.*, 2019). The ability of crops and other commercial plants to adapt to these extreme and stressful conditions is therefore an essential aspect of sustainable agriculture throughout the 21st century (Morton and Abendroth, 2017). Some naturally produced secondary metabolites, belonging to the class of indoleamine, have been demonstrated to assist in abiotic stress tolerance in plants, thus allowing for greater growth and ultimately yield under stressful conditions (Arnao and Hernandez-Ruiz, 2014; Lecube *et al.*, 2014; Erland *et al.*, 2015; Kaur *et al.*, 2015; Mukherjee, 2018). One secondary metabolite of interest in this context is melatonin.

Melatonin (N-acetly-5-methoxytryptamine) is an indoleamine produced by cellular organisms from all domains of life (Arnao and Hernandez-Ruiz, 2014; Hardeland, 2015). Melatonin was first discovered in animals in the late 1950's (within a bovine pineal gland). Research has since primarily considered this indoleamine as a hormone in animals (Lerner *et al.*, 1958; Lerner *et al.*, 1959), associated with various physiological processes including circadian rhythm regulation, mood, immunity,

thermoregulation, sexual activity and reproduction (Maronde and Stehle, 2007; Pandi-Perumal *et al.*, 2008; Jan *et al.*, 2009; Hardeland *et al.*, 2012).

Melatonin was first discovered in plants in 1995 (Dubbels *et al.*, 1995; Hattori *et al.*, 1995), and has since has been reported to have key roles associated with abiotic and biotic stress tolerance in plants, as well as plant biostimulation (Arnao and Hernández-Ruiz, 2013; Arnao and Hernandez-Ruiz, 2014; Zhang *et al.*, 2015; Hardeland, 2016). The numerous beneficial roles of melatonin for plants exposed to stressful conditions has led to suggestions that melatonin may potentially have a role in future agricultural practices (Tan *et al.*, 2012). However, understanding the interaction of soil microbes with melatonin is a key factor determining the viability of this possibility, as soil microbial communities have an essential role in plant growth and development (Wall and Virginia, 1999; Yao *et al.*, 2000; Kirk *et al.*, 2004; Wahid *et al.*, 2016). Melatonin has been detected in microbes including various bacteria and fungi, however the roles of this indoleamine in microbes is very poorly researched and little is understood regarding the functional aspects for microbial activity (Manchester *et al.*, 1995; Tilden *et al.*, 1997; Hardeland, 1999; Tan *et al.*, 2014; Liu *et al.*, 2016).

It is difficult to compare studies investigating the responses of plants or microbes to exogenous melatonin, as not all studies report the controls that were used, and whether ethanol or other solvents such as DMSO, required for dissolving the melatonin, were included within control treatments (Arnao and Hernández-Ruiz, 2007, 2013; Weeda *et al.*, 2014). Ethanol (or other solvents), even at low concentrations (e.g. 0.1%), may be experienced as an abiotic stress by some microbes, thus clearly indicating the importance of reporting and standardising the solvent concentration in future studies associated with melatonin (Chatterjee *et al.*, 2006; Wu *et al.*, 2017).

The research conducted in this thesis focuses primarily on responses of soil microbes to melatonin under abiotic stress conditions at individual and community levels. Additionally, the effects of melatonin applied at the site of foliar infection by the cosmopolitan plant pathogen, *Sclerotinia sclerotiorum*, on tobacco (*Nicotiana tacacum*) was also investigated. This literature review will provide a summary of the limited information about melatonin in microbes, followed by details regarding the importance of melatonin in enhancing plant resistance to bacterial and fungal pathogens. The roles of microbes in soil and gut will be introduced, along with research

relating to the effects of melatonin on soil and gut microbial communities. The roles of indoleamines in plants will be subsequently presented, with particular emphasis relating to stress tolerance. A brief overview of the investigations conducted in each of the three major research chapters within this thesis will be provide at the conclusion of this review.

1.2 Melatonin & microbes

1.2.1 Microbes synthesising melatonin

Melatonin biosynthesis has been described in various genera of bacteria such as *Agrobacterium, Bacillus, Variovorax, Pseudomonas* (Jiao *et al.*, 2016; Ma *et al.*, 2017), *Erythrobacter* and *Rhodospirillum* (Manchester *et al.*, 1995; Tilden *et al.*, 1997); as well as several cyanobacteria and algae (Tan *et al.*, 2010; Byeon *et al.*, 2013; Manchester *et al.*, 2015) (Table 1.1). In filamentous fungi, melatonin has been described in ascomycetes such as *Neurospora crassa* (Hardeland, 1999) and *Trichoderma* spp. (Liu *et al.*, 2016); and basidiomycetes including *Agaricus, Cantharellus, Lactarius* and *Leccinum* (Muszyńska *et al.*, 2011). Melatonin is produced by yeast (*Saccharomyces cerevisiae*), and a recent study has subsequently reported the melatonin biosynthetic pathway associated with *S. cerevisiae* (Figure 1.2) (Sprenger *et al.*, 1999; Vigentini *et al.*, 2015; Germann *et al.*, 2016). However, relative to plants or animals, very little is known about the roles of melatonin in microbes (Tan *et al.*, 2014; Liu *et al.*, 2016).

1.2.2 Responses of microbes to exogenous melatonin

Some investigators have reported antimicrobial properties of melatonin applied exogenously: Melatonin (130-530 µM) was shown to inhibit the *in-vitro* growth of the human bacterial pathogens; *Staphylococcus aureus, Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Tekbas *et al.*, 2008). The growth of *Streptococcus agalactiae* was also inhibited by exogenous melatonin (2 µg / ml) (Atroshi *et al.*, 1998). Higher concentrations (1300 µM) of melatonin have also been reported to inhibit the *in-vitro* growth of the human pathogenic yeast, *Candida albicans* (Öztürk *et al.*, 2000). However, in contrast, an increase in the *in vitro* growth rate of the commensal bacterium, *Entereobacter aerogenes*, that resides within the human intestinal tract, was observed at low melatonin concentration (1 nM) (Paulose and Cassone, 2016). This could be interpreted as indicating that responses by some bacteria to melatonin may be

concentration dependent, however research is required to investigate this matter further. Melatonin has not been reported to have the same degree of impact on *in-vitro* grown plant pathogenic, filamentous fungi. Melatonin had no effect on the *in-vitro* radial growth of *Physalospora piricola, B. cinerea or Mycosphaerella arachidicola* (Wang *et al.*, 2001), while inhibitory *in-vitro* growth effects of melatonin towards a plant pathogen, *Alternaria* spp., were only detected at extremely high concentrations (4000 µM) (Arnao and Hernández-Ruiz, 2015). Melatonin (0.5 – 5 mM) also showed bio-oomyceticide properties by impacting cell viability, virulence intensity and *in vitro* growth of *Phytophthora nicotianae* (Zhang *et al.*, 2018).

In contrast, *in vitro* growth of plant pathogen *B. cinerea* and *F. oxysporum* were unaffected by melatonin at these concentrations (Zhang *et al.*, 2018). Melatonin (10 µM) enhanced the survival of yeast (*Saccharomyces cerevisiae*) against the cytotoxic effects of the amphipathic protein α-synuclein (Zampol and Barros, 2018). Interestingly, endogenous melatonin levels for *Trichoderma asperellum* increased up to three-fold upon exposure to chemical stressors such as cadmium (3 mM $CdCl_2$) and salt (1% NaCl), suggesting that melatonin may have an important role in *Trichoderma* spp. associated with abiotic stress tolerance (Liu *et al.*, 2016). Similarly, endogenous levels of melatonin have also been demonstrated to increase during fermentation process in *Saccharomyces cerevisiae,* potentially as a strategy to enhance tolerance to the increased ethanol production (Rodriguez-Naranjo *et al.*, 2012). Collectively these studies suggest that melatonin may confer a tolerance to abiotic stresses for microbes, similar to the roles observed in plants. Interestingly, melatonin has also been reported to assist resistance by plants to bacterial and fungal pathogens (Section 1.3).

Table 1.1: Examples of melatonin-producing microbes and concentration of melatonin synthesised endogenously or secreted.

Microbe	Melatonin levels	Reference	Microbe	Melatonin levels	Reference
Gram positive bacteria			**Basidiomycetes**		
Bacillus amyloliquefaciens SB-9	Secreted 0.87 ng/mL	(Jiao *et al.*, 2016)	*Boletus edulis*	0.68 mg / 100 g DW	(Muszyńska *et al.*, 2011; Muszyńska and Sułkowska-Ziaja, 2012)
Bacillus thuringiensis CS-9	Secreted 0.53 ng/mL		*Cantharellus cibarius*	0.14 mg / 100 g DW	
			Lactarius deliciosus	1.29 mg / 100 g DW	
Gram negative bacteria			*Agaricus bisporus*	0.11 mg / 100 g DW	
Agrobacterium tumefaciens CS-30	Secreted 0.22 ng/mL	(Jiao *et al.*, 2016)	*Leccinum rufum*	0.08 mg / 100 g DW	
Pseudomonas fluorescens RG11	Secreted 1.32 ng/ml	(Ma *et al.*, 2017)			
Erythrobacter longus	1.914 ng/mg protein	(Tilden *et al.*, 1997)	**Ascomycetes**		
Rhodospirillum rubrum	Not specified	(Manchester *et al.*, 1995)	*Trichoderma spp.*	27.58 – 11.55 µg / g DW	(Liu *et al.*, 2016)
			Saccharomyces uvarum	Not specified	(Rodriguez-Naranjo *et al.*, 2012)
Cyanobacteria					
Synechocystis sp. PCC 6803	Not specified	(Byeon *et al.*, 2013)	*Saccharomyces cerevisiae*	1-100 ng/mg protein	(Sprenger *et al.*, 1999)
Single cell flagellate			**Algae**		
Euglena gracilis	Not specified	(Hardeland, 1999)	*Chondrus crispus*	Up to 3ng/mg protein	(Hardeland, 1999)
			Palmaria palmata	0.1 – 0.4 ng/mg FW	(Lorenz and Liining, 1999)
Dinoflagellate			*Pterygophora californica*	1.5 ng/mg protein	(Fuhrberg *et al.*, 1996)
Gonyaulax polyedra	50 ng/mg protein	(Fuhrberg *et al.*, 1997)			

DW: Dry weight; FW: Fresh Weight.

1.3 Melatonin enhances resistance in plants against bacterial and fungal pathogens

Melatonin has been found to improve resistance for plants against pathogen attack, in part due to an effect on the expression of genes associated with salicylic acid (SA). SA is a phytohormone, acting as an endogenous defence signalling molecule associated with pattern triggered immunity (PTI) and effector-triggered immunity (ETI) in plants (Delaney *et al.*, 1994; Vlot *et al.*, 2009). In particular, SA is a key signalling molecule regulating pathogenesis related (PR) gene expression during systemic acquired resistance (SAR), a process involving the long term resistance of a plant to an individual pathogen (Sorahinobar *et al.*, 2016). Melatonin has been shown to positively affect biosynthesis of salicylic acid in *Arabidopsis* (Shi *et al.*, 2015a). Exogenous melatonin (10 µM) enhanced expression of pathogenesis related (PR) genes including genes associated with salicylic acid biosynthesis in both *Arabidopsis* and tobacco leaves exposed to a bacterial pathogen, *Pseudomonas syringae* pv. tomato DC3000 (Lee *et al.*, 2014). Reduced endogenous melatonin levels (from ~ 4 ng g^{-1} fresh weight (FW) to ~ 3 ng g^{-1} FW) in *Arabidopsis* SNAT KO mutants, resulted in reduced salicylic acid levels and less resistance to pathogenic attack by the avirulent *P. syringe* pv. tomato DC3000 (Lee *et al.*, 2015). Endogenous levels of melatonin in *Arabidopsis* leaves infected with *P. syringae* pv. tomato (Pst) DC3000, were significantly ($p < 0.05$) increased (from approx. 0.5 ng g^{-1} FW to 1 ng g^{-1} FW) only 1 hr post infection, with resistance to infection also increased upon the pre-treatment with 20 µM melatonin (Shi *et al.*, 2015a). Root pre-treatment of apple [*Malus prunifolia* (Willd.) Borkh. cv. Donghongguo] with 100 µM melatonin resulted in enhanced immunity to the foliar fungal pathogen, *Diplocarpon mali* (Figure 1.1) (Yin *et al.*, 2013). Interactions between plant and pathogen induce a rapid oxidative burst in the plant cells/tissues at the early stages of infection, resulting in increased endogenous melatonin levels (Arnao and Hernández-Ruiz, 2015). A recent study found that exogenous melatonin (200 mg/L) applied to leaves reduced the lesion length of rice bacterial leaf streak (BLS) disease, caused by *Xanthomonas oryzae* pv. *oryzicola* (Xoc) by up to 23% on rice strains susceptible to the bacterium. Similarly, foliar pre-treatment with melatonin significantly reduced infection capability and reduced the incidence of BLS by 17% (Chen *et al.*, 2019).

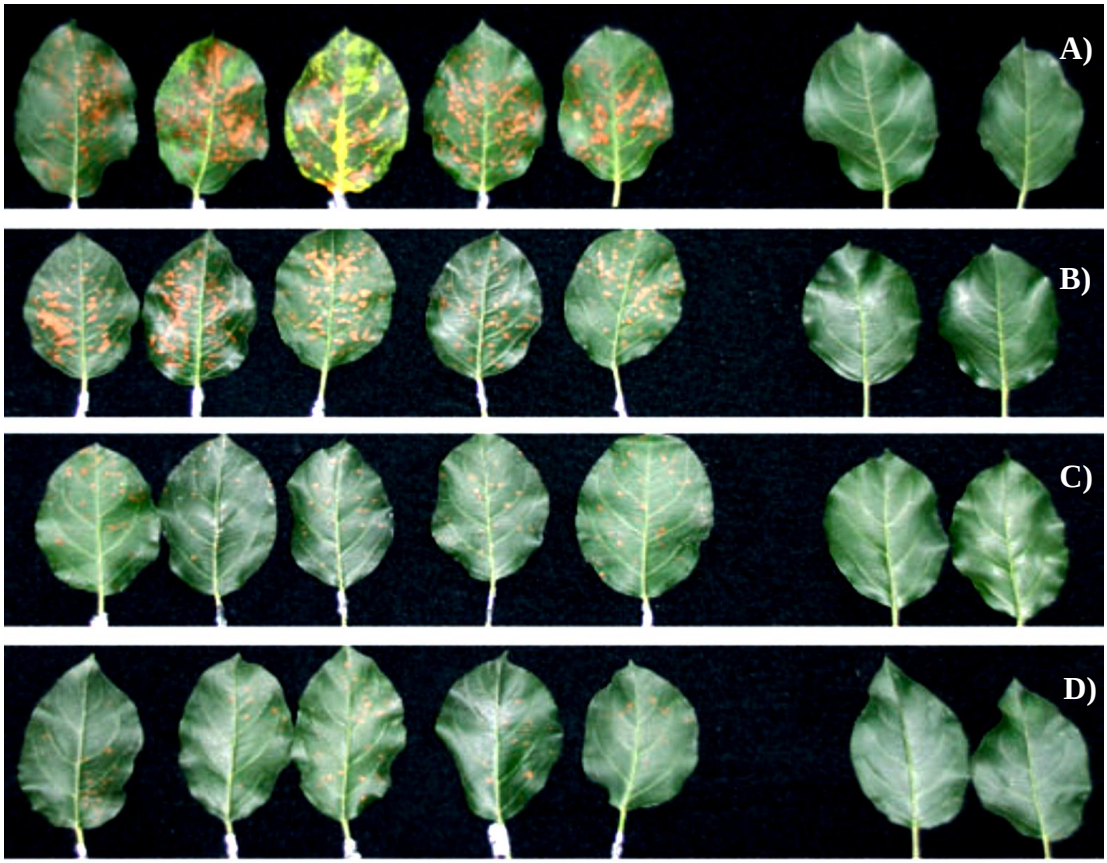

Figure 1.1: Effect of melatonin concentration on apple leaf phenotype at 20 days post infection. Fully mature leaves were collected from plants pretreated (through soil treatment) with water only and no exogenous melatonin (A); 50 µM melatonin (B); 100 µM melatonin (C); or 500 µM melatonin (D). Taken from Yin *et al*., (2013).

Melatonin has also been found to enhance immunity in an insect vector of a plant pathogen. Citrus greening disease is caused by the bacterium, *Candidatus Liberibacter asiaticus* (CLas) and is transmitted by Asian citrus psyllid, *Diaphorina citri.* CLas infection reduced the endogenous levels of melatonin in *D. citri*, compared to uninfected insects (Nehela and Killiny, 2018). However, treatment of *D. citri* with 500 mg/L melatonin resulted in a significant ($p < 0.05$) decrease of CLas bacterial population within the *D. citri* psyllids and enhanced longevity of infected *D. citri* (Nehela and Killiny, 2018).

1.4 Microbial communities in biological systems

1.4.1 Roles of soil microbiome communities

In soils, the predominant bacterial phyla include *Proteobacteria, Actinobacteria and Acidobacteria, with Bacteroidetes, Firmicutes* and *Verrucomicrobia* present generally in lesser relative abundances (< 5%) (Yadav *et al.*, 2015; Delgado-Baquerizo *et al.*, 2018; Wang *et al.*, 2018; Yu *et al.*, 2019). For fungi, *Ascomycota, Basidiomycota* and *Zygomycota* predominate in soils, with *Chytridiomycota* and *Glomeromycota* acting as minor phyla (< 7%) (Wang *et al.*, 2017b; Yang *et al.*, 2017). Soil microbial communities are a natural and key component of a balanced ecosystem (Wall and Virginia, 1999; Yao *et al.*, 2000; Kirk *et al.*, 2004; Wahid *et al.*, 2016), breaking down organic matter, recycling nutrients, creating soil structure, assisting plant growth as well as controlling pests and diseases (Glick, 2014; van der Heijden *et al.*, 2015; Majeed *et al.*, 2018; Jiang *et al.*, 2019). In particular, nitrogen-fixing bacteria and plant growth-promoting rhizobacteria (PGPR), root-associated ectomycorrhizal fungi (EMF) and arbuscular mycorrhizal fungi (AMF) provide essential moisture, minerals and nutrients to plants in many soil systems, thus maintaining plant health and development (Brussaard *et al.*, 2007; Fierer *et al.*, 2007; Chanda *et al.*, 2016). Nitrogen-fixing bacteria such as *Pseudomonas, Bacillus, Rhizobium* and *Azospirillum* (Igiehon and Babalola, 2018) provide nutrients to plants by converting inert atmospheric N_2 to inorganic nitrogen-based molecules (e.g. NH_4^+) (Pajares and Bohannan, 2016) within root nodules, resulting in enhanced above and belowground plant biomass (Igiehon and Babalola, 2018). PGPRs include *N*-fixing bacteria as well as various other genera such as *Flavobacterium, Chryseobacterium, Achromobacter, Enterobacter, Sinorhizobium, Cradyrhizobium* and *Acetobacter* (Dimkpa *et al.*, 2009; Etesami and Maheshwari, 2018). PGPRs can increase plant growth by solubilising soil nutrients (Glick, 2014) and generating phytohormones like gibberellic acid and auxin (Etesami and Maheshwari, 2018), as well as enhancing tolerance to various abiotic stressors by processes such as sequestering toxic heavy metals in contaminated soils (Glick, 2010) and decreasing stress-related ethylene production in developing roots (Glick, 2004). No study to date has reported the production of melatonin in PGPRs.

EMFs comprised of *Basidiomycetes* and *Ascomycetes,* including *Amhinema, Thelephora, Boletus, Cenococcum, Laccaria, Hebeloma, Paxillus, Suillus* and *Phialophora,* exchange soil minerals with plant roots for various sugars or carbon-

based compounds (Brundrett, 2009; Kumar and Atri, 2018; Mello and Balestrini, 2018). EMF symbiosis is mostly associated with angiosperm and gymnosperm trees (Bonfante and Anca, 2009). In contrast, most terrestrial plants, including many non-brassica crops, associate with AMFs (Choi *et al.*, 2018). This relationship involves a symbiotic association with fungi within the four orders of *Glomeromycota: Glomerales, Archaeosporales, Diversisporales* and *Paraglomerales* (Redecker *et al.*, 2013). Over 70% of the inorganic phosphate required by plants is provided by AMFs (Smith *et al.*, 2003, 2004) in exchange for up to 20% of the carbon requirements for the fungus (Jakobsen and Rosendahl, 1990).

Plants directly regulate microbial community profiles within the rhizosphere by altering the composition of sugars, amino acids and various secondary metabolites, such as indoleamines (e.g. auxins), secreted into the soil as root exudates (Badri and Vivanco, 2009; Dennis *et al.*, 2010). For example, up to 40% of photosynthates present within the roots are released as rhizo-deposits into the soil, resulting in significant increases in microbial densities within the rhizosphere compared to bulk soil (Berendsen *et al.*, 2012). Concurrently, soil microbial community structures may also be affected by numerous other factors including soil physiochemical characteristics and exposure to various abiotic stresses (Badri and Vivanco, 2009; Wood *et al.*, 2016a; Geisseler *et al.*, 2017).

1.4.1.1 Melatonin & soil microbiome

To date, only one study has investigated the responses of the soil microbiome to melatonin (Li *et al.*, 2018). In that study, the effect of exogenous melatonin application (200 µM; applied at 20 day intervals for 6 months) on bacterial and fungal compositions in two soils types associated with horticultural practices (apple orchard and vegetables respectively) were explored using Illumina next generation sequencing (NGS). No details were provided regarding solvent concentrations in either melatonin or control treatments. Bacterial compositions of melatonin-treated soil samples associated with vegetables showed increases in *Chloroflexi* (1.28-fold) and *Gemmatiomonadetes* (1.36-fold) and decreases in *Verrucimicrobia* (1.34%) relative to control samples, whereas melatonin-treated soil samples associated with the apple orchard showed increases in *Firmicutes* (1.5-fold), *Parcubacteria* (1.39-fold) and *Gemmatiomonadetes* (1.35-fold) and decreases in *Bacterioidetes* (0.62%) relative to control samples (Li *et al.*, 2018). Fungal compositions of melatonin-treated soil samples associated with vegetables

showed an increase in *Basidiomycota* (1.29-fold), with a decrease in *Ciliophora* (3.53%), whereas melatonin-treated soil samples associated with the apple orchard showed increases in *Ciliophora* (2.28-fold), *Basidiomycota* (1.78-fold) and *Glomeromycota* (4.76-fold), with a decrease in *Ascomycota* (14.44%) relative to control samples (Li *et al.*, 2018). This study demonstrated that melatonin altered bacterial and fungal community compositions in soil and the community shifts in response to melatonin varied between the soils. Therefore, some of the various ecological functions associated with the microbial community in a soil may be affected by application of exogenous melatonin. A clear understanding of how soil microbes are impacted by melatonin is therefore essential in order to determine if there is a potential role for melatonin in future agricultural practices. A weakness of this study however was that only subsoils (20 – 30cm depth) were investigated, which have distinctly different microbial community compositions and activities compared to topsoils (< 10cm) (Schnecker *et al.*, 2015; Sosa-Hernández *et al.*, 2018).

1.4.2 Melatonin & gut microbiome

The gut microbiome consists of microbes, including bacteria, fungi, viruses and archaea residing within the gastrointestinal tract (Hoffmann *et al.*, 2013; D'Argenio and Salvatore, 2015). The main research investigating microbial community responses to melatonin has been associated with the gut microbiome, with studies demonstrating that melatonin impacts diversity and community structures of microbial assemblages (Xu *et al.*, 2017; Ren *et al.*, 2018; Yin *et al.*, 2018; Zhu *et al.*, 2018). However, gut microbiome responses to melatonin are not within the scope of the research conducted in this thesis.

1.4.3 Effects of abiotic stresses (cadmium and salt) on soil microbes

In Chapter 2 of this thesis, the impacts of cadmium and salt stress on soil microbial community structures in the presence of melatonin are investigated. A comprehensive meta-analysis of bacterial communities from 111 different studies found salinity as the most influential determinant of bacterial community structure, ahead of other key abiotic factors such as temperature or pH (Lozupone and Knight, 2007). Seasonally changing salinity also altered the bacterial and fungal community structures of root microsymbionts associated with alder (*Alnus glutinosa*) (Thiem *et al.*, 2018). Similarly, cadmium (> 4 mg kg^{-1}) has been shown to alter bacterial (Wood *et al.*, 2016b; Hu *et al.*, 2018), and fungal (4 mg kg^{-1}) (Shentu *et al.*, 2008) community compositions in various soil studies. For example, soil bacteria such as *Actinobacteria* and

Planctomycetes, are more impacted by cadmium toxicity (8 mg kg^{-1}) compared to other bacterial phyla (e.g. *Acidobacteria* and *Proteobacteria*) (Hu *et al.*, 2018). Plant growth promoting rhizobacteria (PGPRs) such as *Enterobacter* spp. (Chen *et al.*, 2010) and *Pseudomonas spp.* (Sinha and Mukherjee, 2008) are tolerant to 1.5 mM and 8 mM cadmium in soil respectively. *Basidiomycota* showed a positive correlation with soil cadmium levels in heavy metal contaminated soil, increasing in relative abundance with increasing cadmium levels (Jia *et al.*, 2018).

Alteration of microbial communities by plants via root exudate or by exposure to abiotic stresses can have both direct and indirect effects on the overall soil microbial diversity and plant productivity (Berendsen *et al.*, 2012; Rincon-Florez *et al.*, 2013; Chanda *et al.*, 2016). Thus, exploring novel avenues to control stress-induced shifts in soil microbial community composition and functionality could be a key to unfolding agricultural constraints, and achieve better agricultural productivities and sustainable development in the future.

1.5 Soil microbial community analysis

1.5.1 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The study of microbial community diversity requires the use of molecular techniques as many bacteria and fungi found in soil are not culturable (Van Elsas *et al.*, 2000). Automated Ribosomal Intergenic Spacer Analysis (ARISA) is commonly used to characterise microbial community diversity within various environments, including bulk soil and the rhizosphere (Sørensen *et al.*, 2009; Rincon-Florez *et al.*, 2013). ARISA fingerprints fungal and bacterial communities based upon the length heterogeneity of the intergenic spacer region between the ribosomal Ribonucleic Acid (rRNA) genes for bacteria (16S and 23S) and fungi (18S and 28S) (Kirk *et al.*, 2004). This molecular technique provides a valuable estimate of both species richness and relative abundances, allowing a comparison of species richness (α diversity) and community structure (β diversity) both across various sites and between samples exposed to different treatments (Zancarini *et al.*, 2012). ARISA is quick, relatively inexpensive, and has a high level of sensitivity. However taxa are not identifiable, and some rare biases can exist as either unrelated organisms may share the same spacer length, and thus result in an underestimation of species richness, or a single organism may provide more than one peak and therefore result in an overestimation (Rincon-Florez *et al.*, 2013). In general, ARISA is appropriate for soils containing moderate to

low species richness as significant errors in determining microbial richness occur in highly diverse ecosystem samples (e.g. Organosols from rainforest soils) where richness exceeds approximately 150 species (Kovacs *et al.*, 2010).

1.5.2 Next Generation Sequencing (NGS)

Over the past decade, the development of high-throughput NGS technology has greatly benefited our understandings relating to microbial ecology within numerous terrestrial and aquatic ecosystems (Thompson *et al.*, 2017). In particular, amplicon-based DNA sequencing of taxonomic marker genes generates in-depth characterisation of microbial communities. This allows for the monitoring of rare or poorly characterised individuals to dominant taxa under natural conditions and in responses to various treatments (Staley and Sadowsky, 2018). Amplicon sequencing is relatively inexpensive, however sensitivity is restricted by numerous factors including the quality of sequencing technology as well as the relatively short amplicon length (approx. <1000 bp) (Sogin *et al.*, 2006). To reduce errors due to the latter, reliable identification of large datasets of taxa may require classification of microbes to family, order or even phyla level (Mizrahi-Man *et al.*, 2013).

1.6 Reactive oxygen species (ROS)

1.6.1 Roles and effects of ROS (microbes and plants)

ROS are unstable and highly reactive molecules or free radicals containing oxygen, produced during either photosynthesis or metabolism by organisms from all kingdoms of life (Mittler, 2002; Lushchak, 2005; Liemburg-Apers *et al.*, 2015; Inupakutika *et al.*, 2016). ROS molecules include hydrogen peroxide (H_2O_2), nitric oxide (NO) and hypochlorous acid (HClO); ROS radicals include superoxide anion ($O_2^{-\bullet}$), hydroxyl ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and alkoxyl ($RO^{\bullet}$) (Felício *et al.*, 2009; Manchester *et al.*, 2015). At moderate levels, ROS are positively associated with various natural roles such as signal transduction pathways (Baxter *et al.*, 2014; Schieber and Chandel, 2014). However, when exposed to a stress, various activities within the electron transport system are negatively impacted, resulting in dramatic increases in cellular levels of ROS (Foyer *et al.*, 1997; Schieber and Chandel, 2014; Hardeland, 2015). Enhanced levels of ROS result in a multitude of negative impacts at a cellular level including damage to DNA and cell membranes, as well as protein denaturation, reduced enzyme activity and cellular apoptosis (Foyer and Noctor, 2005; Felício *et al.*, 2009; Bose *et*

al., 2014). Some plants produce ROS at the site of infection as a means of defense against pathogenic attack (Ali *et al.*, 2018). ROS can impair bacterial growth as well as shape microbial community structures (Arsenijevic *et al.*, 2000; Imlay, 2019). Production of ROS-scavenging enzymes is an essential stress response mechanism for aerobic microbes (Seaver and Imlay, 2001). Various antioxidants such as glutathione and ascorbate as well as enzymes including catalase (CAT), glutathione reductase (GRT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and peroxidase (POD) restore ROS homeostasis under stressful conditions by scavenging excess ROS (Apel and Hirt, 2004; Ammendola *et al.*, 2008).

1.6.2 Effects of melatonin on cellular ROS

Melatonin can act as a highly efficient antioxidant, with the ability to scavenge up to 10 ROS per molecule (Hardeland, 1999; Reiter *et al.*, 2015; Reiter *et al.*, 2016). Melatonin may also act as a signalling molecule, resulting in the upregulation of ROS-scavenging enzymes or stimulating various metabolic pathways involved in maintaining cellular homeostasis (Rodriguez *et al.*, 2004; Weeda *et al.*, 2014; Zhang *et al.*, 2015). Interestingly, studies comparing the effects of different melatonin concentrations indicate that this secondary metabolite can have an inhibitory effect on at high concentrations in plants, potentially due to the excessive removal of the cellular ROS, thus negatively affecting ROS-dependant signalling pathways essential for natural plant development (Chen *et al.*, 2009; Baxter *et al.*, 2014; Zhao *et al.*, 2015).

Numerous studies in plants have shown that melatonin mitigates the negative impact of excess ROS production caused by stress exposure, by affecting the genes associated with various signalling cascades. For example, melatonin affects stress associated calcium-dependant signalling, a key signalling molecule involved in plant growth and development (Beilby *et al.*, 2014). Melatonin can both enhance the activities of ROS-scavenging enzymes as well as enhance the upregulation of transcription levels of genes associated with stress tolerance. For example, melatonin causes an upregulation of ROS-scavenging enzymes SOD, CAT, APX and glutathione peroxidase (GPX), as well as increasing the activities for these enzymes (Rodriguez *et al.*, 2004; Manchester *et al.*, 2015). ROS scavenging antioxidants such as ascorbic acid (vitamin C), vitamin E and glutathione are also upregulated by melatonin (Zhang *et al.*, 2015).

Melatonin has been demonstrated to upregulate many genes associated with mitigating the negative abiotic stress responses such as ion homeostasis (NHX1 and AKT1) under salinity stress; C-repeat binding factors (CBF's); drought responsive element binding factors (DREB's); cold tolerance gene (COR15a) and transcription factor (CAMTA1); transcription factors associated with ROS antioxidant genes (ZAT10 and ZAT12) (Bajwa *et al.*, 2014). Stress related transcription factors such as zinc finger proteins, WRKY and NAC domain-containing proteins are all upregulated by melatonin (Zhang *et al.*, 2015).

1.7 Melatonin biosynthetic pathway

Melatonin is a highly mobile secondary metabolite, passing easily across cellular and subcellular membranes due to amphiphilic properties (Shida *et al.*, 1994). The first step of the melatonin biosynthetic pathway involves the synthesis of tryptamine from tryptophan by the enzyme tryptophan decarboxylase (TDC). Subsequently tryptamine is converted into serotonin by tryptamine-5-hydroxylase (T5H). Acetylation of serotonin by serotonin *N*-acetlytransferase (SNAT) produces *N*-acetylserotonin (NAS), which is converted into melatonin by either acetylserotonin *O*-methyltransferase (ASMT) or caffeic acid *o*-methyltransferase (COMT) (Figure 1.2) (Kang *et al.*, 2011; Byeon and Back, 2015; Back *et al.*, 2016; Wang *et al.*, 2019). Alternative biosynthetic pathways for serotonin and melatonin in plants are shown in Figure 1.2. For example, 5-methoxytryptamine can be synthesised from serotonin by ASMT, which is then converted to melatonin by SNAT (Nawaz *et al.*, 2016). All pathways involve the presence of tryptophan at the first step and serotonin as an intermediate, indicating the importance of both indoleamines in melatonin biosynthesis in plants and yeast (Back *et al.*, 2016; Germann *et al.*, 2016)

The decarboxylation of tryptophan by TDC is considered the regulatory step in the biosynthesis of serotonin (Byeon *et al.*, 2014b; Hardeland, 2016). However, as the yield of melatonin is relatively an order or more of magnitude less than serotonin, the final two steps are key determinants of melatonin production in plants (Murch *et al.*, 2009; Nguyen Le *et al.*, 2013; Byeon *et al.*, 2014b). Overall, the rate-limiting step in melatonin biosynthetic pathway in plants and yeast is considered to be the methylation of *N*-acetylserotonin to melatonin (Byeon *et al.*, 2014b; Germann *et al.*, 2016; Wang *et al.*, 2019).

Some enzymes involved in the melatonin biosynthetic pathway may have more than one substrate. For example, TDC has a substrate affinity for both tryptophan and 5-hydroxytryptophan (Park *et al.*, 2008; Park *et al.*, 2011). Serotonin and 5-methyoxytryptamine can act as substrates for SNAT, while ASMT has affinity for both serotonin and *N*-acetylserotonin as substrates (Back *et al.*, 2016). Serotonin biosynthesis occurs in the endoplasmic reticulum when the pathway involves the conversion of tryptamine to serotonin by T5H, however the alternative pathway involving TDC occurs in the cytoplasm (Figure 1.2) (Back *et al.*, 2016). Both SNAT and ASMT are expressed in the chloroplast (Byeon *et al.*, 2014a) as well as being localised either in the mitochondria (SNAT) and cytoplasm (ASMT/COMT) (Back *et al.*, 2016; Wang *et al.*, 2017a).

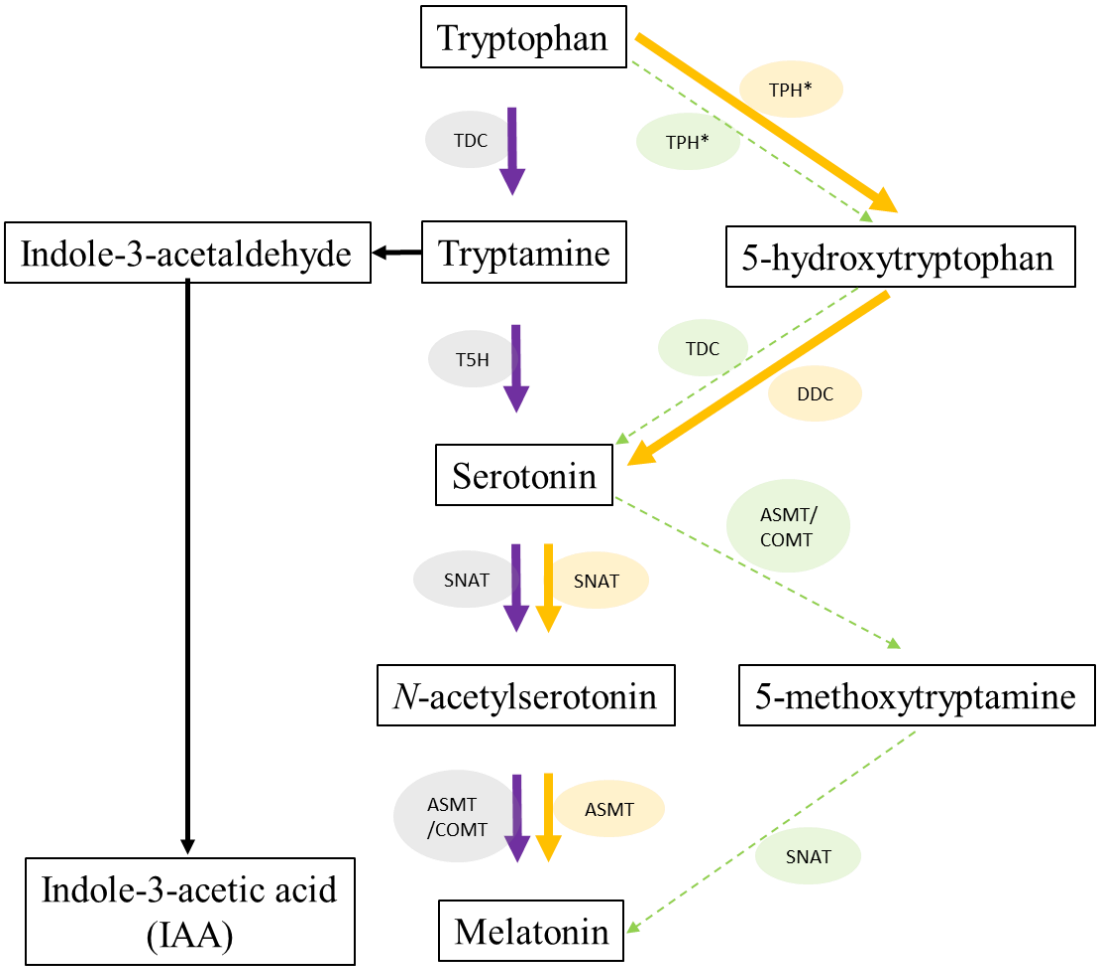

Figure 1.2: Melatonin biosynthetic pathways in yeast and plants (Back *et al.*, 2016; Germann *et al.*, 2016). Arrows and enzymes associated with yeast are labelled in yellow, whereas all remaining arrows and enzymes are associated with plants. Solid purple lines represent the biosynthetic pathways of serotonin and melatonin in plants,

with dotted lines representing alternative pathways (Back *et al.*, 2016; Nawaz *et al.*, 2016). Tryptophan decarboxylase (TDC); L-tryptophan hydroxylase (TPH); 5-hydroxy-L-tryptophan decarboxylase (DDC); Tryptamine 5-hydroxylase (T5H); Serotonin *N*-acetyltransferase (SNAT); *N*-acetylserotonin methyltransferase; Caffeic acid *O*-methyltransferase (COMT) * TPH has yet to be identified in yeast and plants.

When the final step is associated with SNAT, melatonin is synthesised in the chloroplasts, whereas melatonin is produced in the cytoplasm when ASMT/COMT is the terminal enzyme (Back *et al.*, 2016). For example, a dose dependant production of melatonin was observed in purified *Arabidopsis* chloroplasts supplied with *N*-acetylserotonin, (Zheng *et al.*, 2017), while isolated *Malus zumi* mitochondria supplied with serotonin also showed enhanced melatonin levels (Wang *et al.*, 2017a).

The enhanced availability of precursors within the melatonin biosynthetic pathway results in significantly different amounts of melatonin subsequently produced. For example, enhanced levels of exogenous tryptophan did not result in a similar increase in endogenous melatonin levels in *Viola* leaves (Kim *et al.*, 2011). However, upon exogenous treatment with tryptamine, the rate of melatonin biosynthesis increased dramatically (Kim *et al.*, 2011). As melatonin and the phytohormone auxin [Indole-3 acetic acid (IAA)] both share the same precursor, tryptophan (Figure 1.2), exogenous application of tryptophan may have been directed towards the biosynthetic pathway of IAA (or similar molecules) in preference to melatonin. Similarly, melatonin levels decreased in transgenic rice overexpressing a key enzyme in the serotonin biosynthetic pathway, T5H (Fujiwara *et al.*, 2010; Park *et al.*, 2013). The enhanced levels of serotonin resulting from T5H overexpression may have affected and subsequently altered the dynamics within the associated signalling pathways, thus redirecting the emphasis of melatonin biosynthesis. Furthermore, an under expression of the T5H enzyme resulted in an increase in tryptophan and 5-hydroxytryptophan, but not melatonin (Park *et al.*, 2012). In contrast, rice seeds overexpressing TDC resulted in an increase in endogenous melatonin levels 31-fold greater than wild type seeds (Byeon *et al.*, 2014b). Melatonin levels in SNAT KO mutant plants of *Arabidopsis* showed significant decreases in endogenous melatonin, indicating the importance of the enzyme in the biosynthetic pathway. (Lee *et al.*, 2015). Overexpression of ASMT in *Arabidopsis* resulted in increased endogenous melatonin levels (Zuo *et al.*, 2014), while suppression of COMT in rice and *Arabidopsis* resulted in significant reduction in

melatonin levels (Byeon *et al.*, 2015). These results indicate the importance of TDC, SNAT and ASMT/COMT enzymes in melatonin biosynthesis as indicated by their presence in all proposed biosynthetic pathways in Figure 1.2. Importantly, when melatonin is localised in either the chloroplast or cytoplasm it can be metabolised into various substrates including 2-hydroxymelatonin and cyclic 3-hydroxymelatonin respectively, thus reducing endogenous melatonin levels (Byeon and Back, 2015; Lee *et al.*, 2016).

A recent study reported the first discovery of a melatonin receptor in plants, (CAND2/PMTR1) (Wei *et al.*, 2018). In this study, melatonin was found to regulate stomatal closure of a G protein-coupled receptor in the plasma membrane through H_2O_2- and Ca^{2+}- mediated signalling pathways in *Arabidopsis*. This discovery will initiate a new area of research exploring the association between melatonin-activated receptor(s) signalling and stress tolerance in plants.

1.8 Melatonin & abiotic stress tolerance in plants

Numerous research articles have shown a clear association between melatonin and amelioration of some of the negative impacts caused by exposure to abiotic stresses including heat, drought, cold, salt and heavy metals (Table 1.2). Heat shock can impact plant growth and development by enhancing electrolyte leakage and cellular ROS levels as well as reducing chlorophyll content and germination rates (Tiryaki and Keles, 2012; Alam *et al.*, 2018). Drought exposure in plants results in enhanced osmotic and oxidative stresses (Wang *et al.*, 2003). Cold stress negatively affects plant physiology, as cell walls and particularly cell membranes are vulnerable to the destructive effects of cold stress (Kratsch and Wise, 2000; Zhang *et al.*, 2015). Plant growth and development are greatly impacted in salt-stressed soils due to water stress and ion toxicity, resulting in enhanced osmotic pressures and disruption to photosynthesis, protein synthesis and lipid metabolism (Zhang and Blumwald, 2001; Zhu, 2001; Munns and Tester, 2008; Li *et al.*, 2012). Toxic heavy metals can both interact with ligands and compete with essential metals at their binding sites (Bruins *et al.*, 2000). The resulting cellular damage due to enhanced ROS production, restriction of enzymatic activities or structural distortion of the cell wall, cell membrane and various proteins can have deleterious effects (Howlett and Avery, 1997; Valko *et al.*, 2005; Chang and Leu, 2011).

Table 1.2: Effects of melatonin in plants exposed to various abiotic stresses. Plants were pre-treated with melatonin prior to stress exposure, unless otherwise stated. Seeds coated with melatonin prior to stress exposure were labelled as 'primed'. MT = exogenous melatonin concentration; WHC = soil water holding capacity; PEG = Polyethylene glycol.

Plant	**MT / Transgenic**	**Stressor**	**Effects**	**Reference**
Heat				
Arabidopsis	20 µM	45°C; 90 mins	Upregulation of heat shock transcription factors (HSF's),	(Shi *et al.*, 2015b)
Tall fescue *Festuca arundinacea* Schreb.	20 µM	42°C; 6 hr	Reduced leaf electrolyte leakage Increased chlorophyll content	(Alam *et al.*, 2018)
Rice *Oryza. sativa*	Overexpressing SNAT ASMT	55°C; 1 hr	Increased endogenous melatonin levels Enhanced seedling fresh weight	(Byeon and Back, 2014)
Drought				
Cucumber *Cucumis sativus* L.	100 µM (primed)	18% PEG; 14 days	Reduced leaf H_2O_2 levels Enhanced seed germination Reduced chlorophyll degradation	(Zhang *et al.*, 2013)
Soybean *Glycine max*	100 µM (primed)	20% WHC; 10 days	Improved plant biomass	(Wei *et al.*, 2015)
Creeping bentgrass (*Agrostis stolonifera*)	20 µM	No water; 14 days	Reduced drought-induced leaf senescence Downregulated chlorophyll-degrading genes	(Ma *et al.*, 2018)

Table 1.2: Contd.

Plants	MT/ Trangenic	Stressor	Effects	Reference
Cold				
Wheat *Triticum aestivum*	1 mM	2ºC; 3 days	Enhanced membrane stability and antioxidant enzyme activity	(Sun *et al.*, 2018)
Rice *Oryza. sativa*	100 µM	12ºC; 6 days	Reduced impacts to photosynthesis and ROS accumulation	(Han *et al.*, 2017)
Mung beans *Vigna radiata*	20 µM (primed)	5ºC; 2 days	Increased root growth Decreased electrolyte leakage	(Szafrańska *et al.*, 2013)
Cucumber *Cucumis sativus* L.	50 µM (primed)	10ºC; 2 days	Improved germination rates	(Posmyk *et al.*, 2009).
Salt (NaCl)				
Crab apple tree *Malus hupehensis* Rehd.	0.1 µM	100 mM; 15 days	Improved photosynthetic rates and chlorophyll content	(Li *et al.*, 2012).
Maize *Zea mays* L.	1 µM	100 mM; 8 days	Enhanced activities of peroxidase (POD) and ascorbate peroxidase (APX) Improved photosynthetic capacity, root and shoot dry weight	(Jiang *et al.*, 2016)
Watermelon *Citrullus lanatus* L.	50 µM	300 mM; 7 days	Enhanced superoxide dismutase (SOD), catalase (CAT) and APX activities Increased chlorophyll content	(Li *et al.*, 2017)

Table 1.2: Contd.

Plants	MT/ Transgenic	Stressor	Effects	Reference
Heavy metals				
Water chickweed *Malachium aquaticum*	200 µM (not pre-treated)	10 mg kg^{-1} $CdCl_2$; 14 days	Improved cadmium uptake in Cd- hyperaccumulator plants	(Tang *et al.*, 2018)
Potato weed *Galinsoga parviflora*	100 µM (not pre-treated)			
Rice *Oryza. sativa*	Overexpressing SNAT	0.2 mM $CdCl_2$; 3 days	Enhanced endogenous melatonin under stress Increased cadmium tolerance	(Lee and Back, 2017)
Tomato *Solanum lycopersicum* L.	100 µM (not pre-treated)	100 µM $CdCl_2$; 14 days	Enhanced shoot and root growth responses	(Hasan *et al.*, 2015)

1.9 Roles of other indoleamines in microbes and plants

1.9.1 Microbes

1.9.1.1 Serotonin

Serotonin is a secondary metabolite originating from the indoleamine tryptophan, and is a key precursor in the melatonin biosynthetic pathway (Figure 1.2). Very limited information is available regarding the synthesis of serotonin by microbes and the associated roles of the indoleamine. Serotonin-producing microbial genera associated with the gut include *Enterococcus, Aeromonas, Citrobacter, Acinetobacter, Listeria, Escherichia* and *Streptococcus* (Roshchina, 2016), while the pathogenic yeast *Candida* and phototrophic bacterium *Rhodospirillum* have also been reported to produce serotonin (Oleskin *et al.*, 1998a, b). However, no studies to date have reported responses of soil microbes to serotonin.

1.9.1.2 Auxin (IAA)

IAA is produced by some soil microbes, in part acting as a signalling molecule, thus affecting plant - microbes and various microbe-microbe interactions (Fu *et al.*, 2015). For soil fungi such as *Cryptococcus flavus* and *Fusarium delphinoides*, IAA has also been demonstrated to have growth promoting properties (Kulkarni *et al.*, 2013). IAA-producing soil fungi can enhance lateral root development, thus indirectly improving arbuscular mycorrhizal symbiosis (Kaldorf and Ludwig-Müller, 2000; Yao *et al.*, 2005). Some fungal pathogens secrete IAA during disease development to enhance infection capability (Reineke *et al.*, 2008). Plants also secrete IAA within root exudates, thus altering microbial community structures within the rhizosphere (Narasimhan *et al.*, 2003; Badri and Vivanco, 2009; Etemadi *et al.*, 2014). As melatonin is structurally similar to IAA and has been shown to have similar effects on plant growth and development, it is possible that melatonin may also be utilised by microbes similar to IAA, however further research is required to explore this possibility.

1.9.2 Plants

1.9.2.1 Serotonin

Serotonin has been reported in a wide variety of plant families, with endogenous levels varying considerably, from almost 400 μg g^{-1} (Butternut (*Juglans cinerea* L.)) to ng or

pg g^{-1} levels, depending on plant family, species and tissue (Kang *et al.*, 2009; Erland *et al.*, 2016). The main site for serotonin biosynthesis is in the roots (Kaur *et al.*, 2015), and the rate limiting step in the biosynthetic pathway is associated with the activity of TDC (Figure 1.2) (Kang *et al.*, 2008). Serotonin is associated with numerous essential roles in plants including senescence, flowering, germination, root and shoot organogenesis, as well as abiotic and biotic stress tolerance (Table 1.3) (Kang *et al.*, 2009; Erland *et al.*, 2015; Erland *et al.*, 2016).

1.9.2.2 Auxin (IAA)

IAA is involved in various essential processes in plants such as cell elongation and division, apical dominance as well as responses to various environmental factors such as light and gravity (Covington and Harmer, 2007; Fu *et al.*, 2015). The main site for IAA biosynthesis is in the roots, however IAA may also be synthesised in the shoot meristem (Gallavotti, 2013; Kaur *et al.*, 2015).

Melatonin and IAA are indoleamines, with common precursors tryptophan and tryptamine in their respective biosynthetic pathways (Figure 1.2) (Arnao and Hernandez-Ruiz, 2014). Structural similarity has led to investigations to determine if particularly melatonin and IAA may be involved in similar processes in plants. Some studies have revealed concentration-dependant developmental effects of melatonin, similar to that of IAA (Hernández-Ruiz *et al.*, 2004; Chen *et al.*, 2009). Overall however, the relationship between melatonin and IAA is currently quite unclear with some reports showing that melatonin alters endogenous IAA levels (Wang *et al.*, 2014; Zuo *et al.*, 2014), while other contradictory studies have reported that melatonin possess its own mechanisms independent of IAA (Pelagio-Flores *et al.*, 2012; Wan *et al.*, 2018).

Table 1.3: Examples of serotonin enhancing abiotic and biotic tolerance in plants. Exogenous serotonin was supplied along with the stressor (i.e. not pre-treated). Enhanced endogenous levels of serotonin were observed in Capsicum (wild type) and rice (harbouring blast resistance gene *Pit*) in response to biotic stress exposure.

Plant	**Serotonin**	**Stressor**	**Effect**	**Reference**
Rice *Oryza sativa* L.	Enhanced endogenous levels	Fungal pathogen	Suppressed lesion development by rice blast fungus *Magnaporthe oryzae*	(Hayashi *et al.*, 2016).
Capsicum *Capsicum annuum* L.	Enhanced endogenous levels	Fungal pathogen	Enhanced resistance to anthracnose disease - *Colletotrichum gloeosporioides* (Penz.)	(Park *et al.*, 2009)
Wheat *Triticum aestivum* L.	1 mM	Fungal pathogen (Detached leaf assay)	Inhibited *in planta* sporulation of *Stagonospora nodorum*,	(Du Fall and Solomon, 2013)
Sunflower *Helianthus annuus* L.	15 µM	120 mM NaCl; 4 days	Enhanced root and hypocotyl growth	(Mukherjee *et al.*, 2014)

1.10 Gaps in research

Melatonin is a highly conserved secondary metabolite, potentially utilised by fungi (filamentous and yeasts) to enhance abiotic stress tolerance (Rodriguez-Naranjo *et al.*, 2012; Liu *et al.*, 2016). Melatonin has antimicrobial properties towards some bacteria, fungi and oomycetes, and has been found to alter microbial community structures in soil and the gut (Atroshi *et al.*, 1998; Tekbas *et al.*, 2008; Xu *et al.*, 2017; Li *et al.*, 2018; Zhang *et al.*, 2018). In plants, melatonin can assist in the amelioration of some of the negative impacts from exposure to abiotic and biotic stresses (Khan *et al.*, 2008; Kang *et al.*, 2009; Park *et al.*, 2009; Khan *et al.*, 2013; Yadu *et al.*, 2017). Melatonin may directly mitigate a stress-induced ROS burst by acting as a highly efficient antioxidant, or mitigate indirectly, by acting as a signalling molecule associated with ROS scavenging enzymes or numerous phytohormones (Hardeland, 1999; Rodriguez *et al.*, 2004; Tan *et al.*, 2012; Weeda *et al.*, 2014; Hardeland, 2015; Reiter *et al.*, 2015; Zhang *et al.*, 2015; Reiter *et al.*, 2016; Arnao and Hernández-Ruiz, 2018). There are no data to date indicating how soil microbial communities respond to melatonin under abiotic stress conditions, and the implications that this may have for the overall health and functioning of the soil. Data is also lacking regarding how plant-microbe interactions may be impacted by exogenously applied melatonin or how endogenous melatonin production may be impacted in either plants or soil-borne microbes by these interactions. Very little is also understood regarding the responses of pathogens to melatonin during infection or disease development phases.

1.11 Thesis aims & hypotheses

This thesis is composed of four main chapters, with chapters 2-4 written as a stand-alone manuscript format (with the exception of overlapping methods across chapters) for submission to a journal for publication. The overall aim of this research was to investigate the responses of soil microbes to melatonin from an individual to community level under unstressed and abiotic stress conditions.

1.11.1 Chapter 2

To date, no studies have reported the effects of melatonin on soil microbial community structures under abiotic stress. This study investigated how melatonin (4 or 0.2 mg kg^{-1} soil) affected total biomass, OTU richness (α diversity) and community structures (β diversity) for bacterial and fungal assemblages in three agricultural soils artificially

stressed with either cadmium (280 or 100 mg kg^{-1} soil) or salt (NaCl 7 or 4 g kg^{-1} soil). Community fingerprinting was conducted by Automated Ribosomal Intergenic Spacer Analysis (ARISA). It was hypothesised that:

1) **melatonin impacts the α and β diversity of microbial communities in soils unstressed and under abiotically stressed conditions**
2) **specific taxa are particularly responsive under the various conditions.**

1.11.2 Chapter 3

Following from the investigation in chapter 2, the effects of melatonin on soil microbial community structures were investigated in greater depth. Taxa responding to exogenous melatonin (4 and 0.2 mg kg^{-1} soil) were identified by next generation sequencing (NGS) in two of the agricultural soils at two different soil sampling timepoints (T0 and T1). Based upon these results, we were able to characterise bacterial groups most responsive (positively or negatively) to treatments. It was hypothesised that:

1) **melatonin alters bacterial community structures in soil**
2) **bacterial communities shift between sampling timepoints for the same soil. This shift in turn results in different responses by soil bacteria to melatonin at both timepoints**
3) **soil microbes respond differently to high and low concentrations of melatonin**
4) **melatonin alters the abundance of soil PGPRs within the soil communities.**

1.11.3 Chapter 4

Research associated with this chapter investigated the *in vitro* growth responses of three fungal plant pathogens (*Sclerotinia sclerotiorum*; *Botrytis cinerea* and *Fusarium oxysporum* f.sp. *vasinfectum*) to melatonin (20 – 4000 µM) under two abiotic stresses (2.5% v/v ethanol and cold (4ºC)). *In planta* growth of *S. sclerotiorum* on *Nicotiana tabacum* leaves infiltrated with melatonin (200 µM) was investigated to determine if the immediate availability of melatonin at the site of infection affected the foliar disease development. It was hypothesised that:

1) ***in vitro* fungal growth under abiotic stress conditions is improved by exogenous melatonin**

2) **the availability of melatonin at the site of infection alters the infection capability of *S. sclerotiorum***

1.11.4 Chapter 5

An overall discussion is presented, linking the major findings and relevance of the research described in chapters 2-4 followed by suggestions for future studies on the basis of these findings.

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Chapter 2 Melatonin shapes microbial community structures in agricultural soils under abiotic stress

2.1 Abstract

An extensive body of evidence from this last decade indicates that melatonin enhances plant resistance to several biotic and abiotic stressors. This has led to an interest in the use of melatonin in agriculture to reduce negative physiological effects from environmental stresses that affect yield and crop quality. However, there are no reports regarding the effects of melatonin on soil microbial communities under abiotic stress, despite the importance of microbes for plant root health and function. Three agricultural soils associated with different land usage histories (pasture, canola or wheat) were placed under abiotic stress by cadmium (280 or 100 mg kg^{-1} soil) or salt (7 or 4 g kg^{-1} soil) and treated with melatonin (4 and 0.2 mg kg^{-1} soil). The effects on microbial community structure were compared to controls using Automated Ribosomal Intergenic Spacer Analysis (ARISA). Significant differences in Operational Taxonomic Unit richness (α diversity) and community structures (β diversity) was observed between bacterial and fungal assemblages across all three soils, demonstrating the effect of melatonin on soil microbial communities under abiotic stress. The analysis also indicated that the microbial response to melatonin is governed by the type of soil and history. The effects of melatonin on soil microbes needs to be regarded in potential future agricultural applications.

2.2 Introduction

Soil microbial communities have an essential role in maintaining ecosystem health by direct exchange of nutrients and minerals with plants within the rhizosphere, as well as by providing nourishment to plants indirectly via nutrient cycling of organic matter (Wall and Virginia, 1999; Yao *et al.*, 2000; Kirk *et al.*, 2004; Wahid *et al.*, 2016). Microbial community structures in soil are altered by various abiotic stresses such as soil contaminants and salinity; as well as various secondary metabolites within root exudates, such as indoleamines (Badri and Vivanco, 2009; Fu *et al.*, 2015; Wood *et al.*, 2016a; Geisseler *et al.*, 2017). Anthropogenic activities, such as phosphorus fertiliser treatment, may introduce the highly toxic heavy metal cadmium (e.g. 300 mg Cd / kg P) into the environment, while irrigation with contaminated water may introduce cadmium or salt(Jiao *et al.*, 2012; Khairy *et al.*, 2014; Bencherif *et al.*, 2015). Both cadmium toxicity and soil salinization cause a dramatic increase in cellular levels of

highly destructive reactive oxygen species (ROS) as well as inhibiting the activities of ROS scavenging enzymes in microbes (Tanaka *et al.*, 2006; Achard-Joris *et al.*, 2007; Hossain *et al.*, 2012; Robertsa, 2014), thus decreasing crop yield. Globally, up to 2 million hectares of agricultural land are negatively impacted by salinization each year (Bencherif *et al.*, 2015; Ke *et al.*, 2017). Alterations to the soil microbial community can have important repercussions in agricultural settings, where microbes (both soil bacteria and fungi) are critical drivers of soil health and agricultural crop productivity (Mau *et al.*, 2019; Zhang *et al.*, 2019).

Melatonin (N-acetly-5-methoxytryptamine) is an indoleamine (secondary metabolite) produced by all cellular organisms (Hardeland, 2015; Manchester *et al.*, 2015), that can decrease the physiological deleterious effect of abiotic stresses. Melatonin can act either as a highly efficient antioxidant, scavenging up to 10 ROS per molecule, or as a signalling molecule regulating enzymes or hormones associated with ROS scavenging (Weeda *et al.*, 2014; Reiter *et al.*, 2015; Zhang *et al.*, 2015; Hardeland, 2016; Reiter *et al.*, 2016). Melatonin exposure alters gut microbiota composition (Schultz *et al.*, 2006; Chen *et al.*, 2011) and may have antibiotic activity against some microbes (Lucchelli *et al.*, 1997). In plants, exogenous melatonin application via seed-coatings (Wei *et al.*, 2015), soil treatments (Cui *et al.*, 2017), or foliar sprays (Zhang *et al.*, 2017) have been described to promote growth and protect plants against stressors such as cadmium (Byeon *et al.*, 2015; Li *et al.*, 2016; Gu *et al.*, 2017) and salt (Li *et al.*, 2012; Liang *et al.*, 2015; Jiang *et al.*, 2016). However, very little is known about the effects of this secondary metabolites on individual microbes or mixed microbial communities in soil (Tan *et al.*, 2014; Manchester *et al.*, 2015; Paulose *et al.*, 2016).

The effects of melatonin on microbial community structures in three different agricultural soils artificially stressed with cadmium or salt were examined. It was hypothesised that: 1) melatonin impacts microbial community structures in soils unstressed and under abiotically stressed conditions; 2) specific taxa are particularly responsive under the various conditions. This is the first study to date to report significant responses of microbes to melatonin under abiotic stress in agricultural soils at a community level.

2.3 Materials & method

2.3.1 Soil sampling and physiochemical characteristics

Three agricultural soils associated with different land uses were collected from sites within Victoria, Australia. Prior to sampling in late March and early April 2017, site "P" (37º32'28.1" S, 145º05'42.5" E) was most recently (< 3 months) associated with cattle and sheep pasture, site "C" (37°45'28.4" S, 144°14'30.2" E) was 3 weeks post canola harvest, and site "W" (37°43'31.1" S, 144°13'14.3" E) was 3 weeks post fire blazing of stubble following a wheat harvest. As the plant species associated with a soil has been shown to be a key factor influencing the bacterial community structure within the rhizosphere (Burns *et al.*, 2015), it was expected that selecting these soils would provide the most diverse range of soil microbes for the current study.

At each site, approximately 4 kg topsoil was sampled (10 cm deep x 0.5 m width x 0.5 length) from each of four plots spaced 3 m apart. Soils were air dried overnight and sieved to remove particles larger than 2 mm. A single stock soil sample was generated for each site by pooling all four collected soils from the subsite plots (Aye *et al.*, 2016; Butterly *et al.*, 2016). The collected stock soils were separately stored at ambient room temperature (21ºC) in airtight plastic containers for four months, followed by subsampling (representing the first sampling timepoint; labelled as 'T0' in the analysis) for immediate treatments as described below. During this first soil subsampling process stock soils were thoroughly aerated. The stock soils were then stored at ambient room temperature (21ºC) in airtight plastic containers for a further four weeks and subsampled for immediate treatments (representing the second sampling timepoint; labelled as 'T1' in the analysis) (Figure 2.1). It was expected that this aging regime would change the initial microbial populations and potentially shift selection pressures amongst the populations (Kelly *et al.*, 1999; Castro *et al.*, 2010; Heijboer *et al.*, 2018; Reese *et al.*, 2018). Physicochemical analyses of untreated soils were conducted by Nutrient Advantage (Melbourne, Australia) (Supplementary Table 2.7.1). Electrical conductivity (EC) of untreated and salt-treated (NaCl) soils was determined using 1:5 soil:water as per He *et al.* (He *et al.*, 2012) (Supplementary Table 2.7.2).

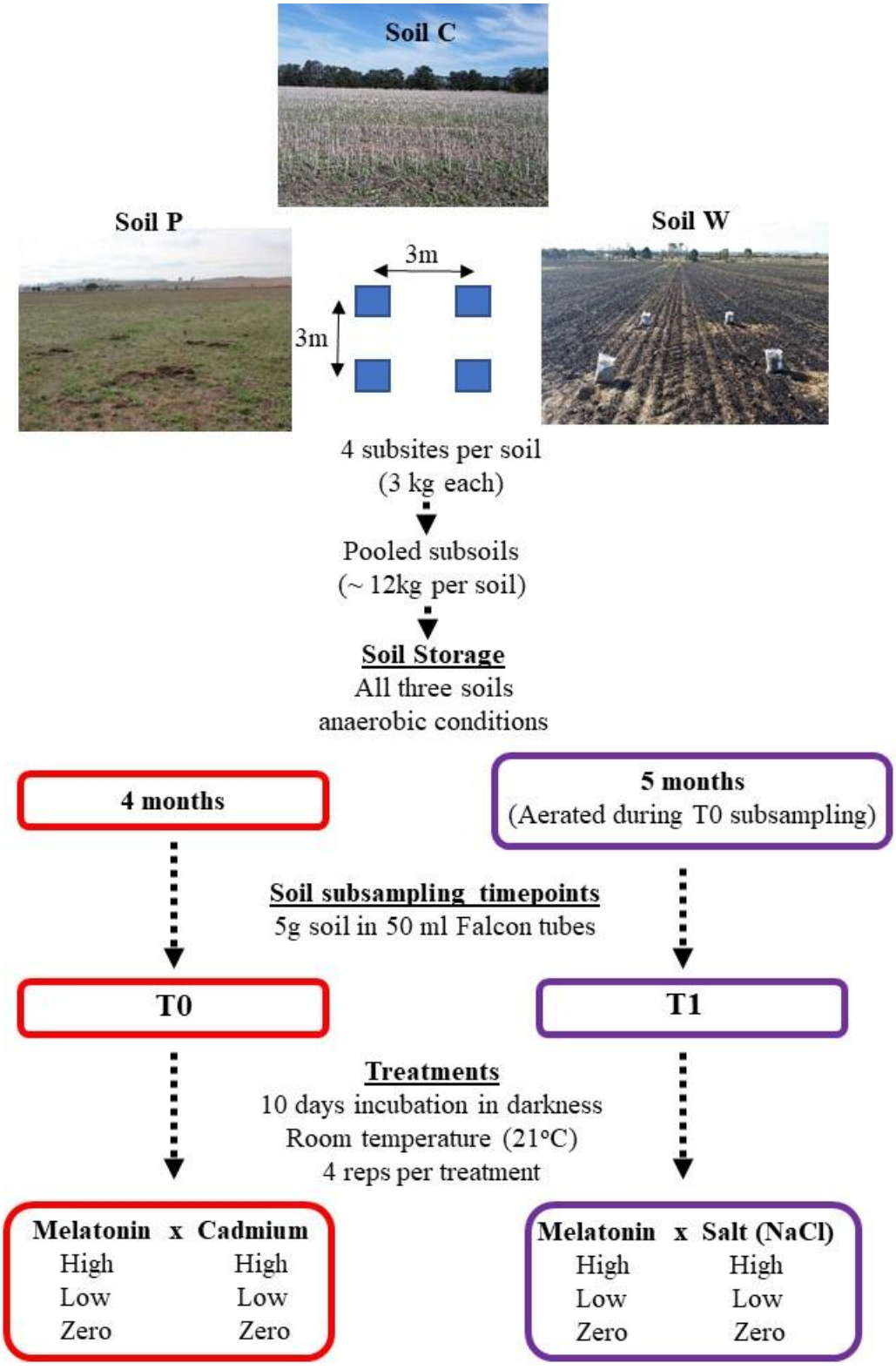


Figure 2.1: Schematic diagram of workflow for preparations of soils P, C and W for treatments at timepoints T0 and T1.

2.3.2 Abiotic stress (cadmium or salt) and melatonin treatments

The responses of soil microbial communities to exogenous melatonin application was investigated with and without cadmium and salt as separately applied stressors (all chemicals from Sigma Aldrich Pty. Ltd., Australia). Five grams of sieved topsoil was transferred to a sterile 50 ml Falcon tube and exposed to various treatment combinations. At sampling timepoint T0, treatments were composed of high or low melatonin (4 or 0.2 mg kg^{-1} soil respectively) and / or high or low cadmium (cadmium chloride hemipentahydrate) (280 or 100 mg kg^{-1} soil respectively) as well as controls. At sampling timepoint T1, treatments were composed of melatonin (4 or 0.2 mg kg^{-1} soil) and / or high or low salt (NaCl) stressor (7 or 4 g kg^{-1} soil respectively). These concentrations were selected to be within the range of those reported to induce effects on soil microbial activities in previous studies for cadmium (Cáliz *et al.*, 2013; Wood *et al.*, 2016b) and salt (Rath *et al.*, 2016). Based upon preliminary studies, soils were treated to 80-90% field capacity, to ensure all soils were sufficiently exposed to stressor and melatonin. Soils without the addition of melatonin or stressor acted as a control.

Melatonin was initially dissolved in 99.9% ethanol to 200 mM and diluted to the required concentrations with sterile Milli-Q water. All treatments and controls contained a standardised amount of ethanol (100 µl of 0.43% ethanol per 5 g soil). Due to differing water holding capacities for each soil, this equated to a final ethanol concentration of 0.044%, 0.052% and 0.06% v/v in treatments within soils P, C and W respectively. Control treatments were composed of dilute ethanol replacing melatonin and sterile Milli-Q water replacing cadmium (timepoint T0) or NaCl (timepoint T1). Treatments and controls were conducted in quadruplicates. Samples were incubated in sterile 50 mL falcon tubes covered with loosely fitted lids to enable gas exchange at room temperature (21^{o}C) in darkness for 10 days. This incubation period was chosen as microbial activity is generally stabilised 7-10 days post stress/moisture exposure (Chowdhury *et al.*, 2011a). Four untreated sample replicates per soil (i.e. no water added) were also collected on Day 0 to provide a representation of the baseline communities prior to treatments.

2.3.3 Microbial community fingerprinting – Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Total soil DNA was extracted from 0.25 - 0.3 g of soil subsamples using PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., California, U.S.A.) according to manufacturer's instructions. ARISA provided a valuable estimate of both OTU richness (α diversity) and relative abundances of OTUs (β diversity), allowing comparisons of α and β diversity both across various soils and between samples exposed to different treatments (Zancarini *et al.*, 2012). Bacterial and fungal community analyses by ARISA were conducted by Australian Genome Research Facility as per Wood *et al.*, (2016b), targeting the intergenic spacer region of the 16S – 23S rRNA genes for bacteria and the internal transcribed spacer (ITS) regions 1 and 2 for fungi (Wood *et al.*, 2016b).

2.3.4 Statistical Analysis

OTU fragment sizes were limited to a range of between 140 – 1000 bp for both fungi and bacteria to ensure only the intergenic spacer region was represented in the data (Fisher and Triplett, 1999). Singletons and low abundance amplicons (<1% relative abundance) were disregarded (Fisher and Triplett, 1999). Data were normalized in the statistical software R version 3.3.2 (The R Foundation for Statistical Computing, Boston, USA), with bin sizes of 3 and 4 selected for bacteria and fungi respectively (Ramette, 2009; Butterly *et al.*, 2016). The following analyses were conducted in Primer-E v6 (Quest Research Ltd., Auckland, New Zealand), with treatments considered as fixed factors and microbial responses analysed for each soil separately: 1) Bray-Curtis similarity for microbial communities; 2) SIMPER analysis to identify the contribution of individual Operational Taxonomic Units (OTUs) to (dis)similarity between replicates or different treatments; 3) DIVERSE to determine Shannon diversity index (H') for sample data; 4) Permutational multivariate analysis of variance (PERMANOVA) to determine treatment effect (melatonin, stressors: cadmium and Salt) on microbial assemblages for each soil at various concentrations (High, Low and Zero) (Chow *et al.*, 2013; Wood *et al.*, 2016b). Monte Carlo statistical analysis was conducted to determine if individual treatment combinations had statistically significant effects on community compositions (Van Wijngaarden *et al.*, 1995). Shannon's diversity index (H') was calculated from binned data to determine differences in biodiversity (relative abundance and evenness) of taxa present within each soil for fungal and bacterial communities upon treatment with melatonin (averaged

for 4 replicates) (Ondreičková *et al.*, 2016). Subsequently, Shannon index values were analysed by non-parametric Wilcoxon test to determine significant ($p < 0.05$) differences upon melatonin treatments under high or low concentration for the stressors (cadmium or Salt). Non-multidimensional scaling (nMDS) and principal coordinates Analyses (PCoA) plots were generated using Analysis of Similarity (ANOSIM) default parameters (significance [$p < 0.05$], 9999 permutations) for visual representation of community (dis)similarities between samples.

2.3.5 Quantitative PCR

Bacterial and fungal abundances were measured by quantitative PCR (qPCR) on DNA of the 16s rRNA and ITS region respectively. Bacterial communities were assessed using primer pairs 1114f (5'-CGG CAA CGA GCG CAA CCC-3') -1275r (5'-CCA TTG TAG CAC GTG TGT AGC C-3'), and fungal communities were assessed using ITS1F (5'-TCC GTA GGT GAA CCT GCG G-3') -5.8Sr (5'-CGC TGC GTT CTT CAT CG-3') primer (Wood *et al.*, 2016b). Each treatment consisted of four biological replicates each quantified in technical triplicate using a CFX Connect Real-Time PCR Detection System (BioRad). A 20 µl reaction mixture for bacterial samples was composed of 2 µl extracted DNA (0.25 ng/µl) and 18 µl Mastermix according to the following recipe: 3.3 µl Universal SYBR® Green Super Mix; 0.27 µl of each 10 µM forward and reverse primer (135 nM final concentration of each primer in reaction mixture); 14.16 µl DNA-free water. Bacterial qPCR reaction mixtures were held at 94°C for 3 min, followed by 40 cycles of amplification at 94°C for 10 sec, 61.5°C for 30 sec. Fungal samples were prepared to a 10 µl reaction mixture composed of 2 µl extracted DNA (0.25 ng/µl) and 8 µl mastermix composed of 4.5 µl Universal SYBR® Green Super Mix; 0.5 µl of each 10 µM forward and reverse primer (500 nM final concentration of each primer in reaction mixture); 2.5 µl DNA-free water. Fungal qPCR conditions were 95°C for 5 min, followed by 40 cycles of amplification at 95°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec. A melting curve was measured from 65°C up to 95°C following qPCR reactions by increasing in 0.5°C increments every 30 sec. Purified amplicons from pure isolates of *E. coli* and *Penicillium* sp. cultures were used to generate standard curves (10-fold series dilutions) for bacterial and fungal samples respectively.

2.4 Results

2.4.1 Melatonin effects soil microbial diversity and abundance across all three soils

Three agricultural soils with different land use practices and diverse physiochemical characteristics were used (Supplementary Table 2.7.1) with the aim of providing the most diverse range of microbial assemblages (Geisseler *et al.*, 2017). A mean of 29.7, 30 and 36 bacterial OTU's, and 16.6, 16.4 and 14.8 fungal OTU's, for untreated soils P, C and W respectively were detected. The ARISA community profiles representing bacterial and fungal communities in the three dry untreated soils were statistically significantly different to each other (Supplementary Figure 2.7.1).

The addition of melatonin to all three soil types had significant effects (increases and decreases) on the bacterial α diversity ($p < 0.05$) but did not significantly affect the fungal α diversity (Supplementary Information 2.7.1). Individual bacterial and fungal OTUs that responded strongly to melatonin showed relative abundances increased by up to 7.1% and 11.5% and reduced by 16.9% and 10.2% respectively (Supplementary Tables 2.7.4 & 2.7.5). Analysis of the soils OTUs compositions (β diversity) by Permutational Multivariate Analysis of Variance (PERMANOVA) indicated that bacterial and fungal community were significantly altered by melatonin and stressors application (Table 2.1, Supplementary Tables 2.7.6 – 2.7.8). The responses of microbial communities in all soils to melatonin under abiotic stress conditions were visualised by non-metric multidimensional scaling (nMDS) ordination (Figures 2.2 & 2.4; Supplementary Figures 2.7.7 & 2.7.8).

Table 2.1: Bacterial a) and fungal b) community responses to treatments (β diversity) with melatonin, stressors and melatonin-stressor combinations based on PERMANOVA analyses of ARISA data (Bray-Curtis dissimilarity distances).

a)

		MT		**Stressor**		**MT x Stressor**	
		pseudo-F	p-value	pseudo-F	p-value	pseudo-F	p-value
Soil P	Cd (T0)	1.429	0.1412	1.516	0.1121	1.975	**0.0195***
	Salt (T1)	2.418	**0.0006***[a]	3.072	**0.0002*****	1.425	**0.046***
Soil C	Cd (T0)	2.151	**0.0213****	9.151	**0.0001*****	0.941	0.546
	Salt (T1)	3.166	**0.0029****	19.587	**0.0001*****	8.721	**0.0001*****
Soil W	Cd (T0)	10.668	**0.0001*****	8.795	**0.0001*****	2.277	**0.0001*****
	Salt (T1)	7.839	**0.0001*****	10.595	**0.0001*****	10.232	**0.0001*****

b)

		MT		**Stressor**		**MT x Stressor**	
		pseudo-F	p-value	pseudo-F	p-value	pseudo-F	p-value
Soil P	Cd (T0)	1.1462	0.2957	2.8194	**0.0008*****	1.123	0.2854
	Salt (T1)	1.4014	0.0811	1.8788	**0.0054****	1.414	**0.0284***
Soil C	Cd (T0)	1.0284	0.4271	2.1996	**0.0071****	1.2995	0.1264
	Salt (T1)	1.1854	0.2517	4.2529	**0.0001*****	1.553	**0.0167***
Soil W	Cd (T0)	4.7926	**0.0001*****	1.8998	**0.035***	0.7398	0.8123
	Salt (T1)	2.1305	**0.0062****	6.0265	**0.0001*****	1.8435	**0.0033****

MT: melatonin; Stressor: cadmium (Cd) or Salt. Sampling timepoints: T0 or T1. All treatments and controls were composed of a standardised amount of dilute ethanol. n=4 replicates per treatment. Significance of PERMANOVA (highlighted in bold): *: 0.01 < p-value ≤ 0.05; **: 0.001 < p-value ≤ 0.01; ***:p-value ≤ 0.001

Under abiotic stress conditions, bacterial communities responding significantly (p < 0.05) to melatonin showed a decreased Shannon diversity index (OTU abundance and evenness), whereas fungal communities increased under the same conditions (Supplementary Information 2.7.4). Overall melatonin had very little effect on bacterial 16S or fungal 18S rRNA copy numbers, with only one soil bacterial community (Soil W) significantly impacted (p < 0.05) by exogenous melatonin only, while fungal communities were unaffected by melatonin-only treatments (Supplementary Information 2.7.5). Some significant differences between treatments were recorded, however no consistent pattern of shift in microbial biomass under stress conditions +/- melatonin was observed (Supplementary Information 2.7.5).

2.4.1.1 Microbial community responses to melatonin treatments

Melatonin had different impacts on bacterial versus fungal composition in soils. High melatonin concentration had a significant effect (PERMANOVA: $p < 0.01$) on bacterial assemblages in all three soils, whereas low melatonin concentration impacted only bacterial communities within soil C and W (Supplementary Table 2.7.6a). Fungi responded less to melatonin compared to bacteria within the same samples. Melatonin had no effect on fungal community structures in soil C (Supplementary Table 2.7.6b), while only high melatonin concentration treatment at sampling timepoint T1impacted fungal community structures in soil P (Table 2.1). However, melatonin at high and low concentration induced shifts in fungal community structures within soil W.

2.4.1.2 Microbial community responses to stressor treatments

Stressors impacted microbial β diversity (Table 2.1). Bacterial community structures in soils C and W were significantly ($p < 0.01$) impacted by treatments with high and low concentrations of cadmium and salt (Supplementary Table 2.7.7a). Similarly, bacterial community structures in soil P were responsive to salt at high and low concentrations, but community structures in this soil were not significantly affected by cadmium. Fungal communities in all soils were impacted by high concentrations of cadmium and salt. In contrast, low concentrations of salt impacted fungal community structures in soil C and W (not P), whereas only fungal communities in soil P were altered by low cadmium treatment (Supplementary Table 2.7.7b).

2.4.1.3 Microbial community responses to melatonin with stressor treatments

In comparison to stressor-only treatments, consistent responses to melatonin were observed in bacterial communities compared to fungal communities, especially at high melatonin concentration (Table 2.2; Supplementary Table 2.7.8). Bacterial responses to high melatonin concentration were significant (in comparison with stressor only treated communities) under high cadmium or salt conditions in soils P and W, but not in soil C (Table 2.2). Under low stressor conditions, bacterial communities in soil C and soil W responded significantly to high melatonin treatment. In contrast, bacterial communities responded to the availability of low melatonin under high stress conditions only in soil W only (both stressors) and under low stressor (salt only) in soils C and W. Fungal communities were far less responsive to melatonin treatments under abiotic stress conditions. In comparison with stressor only treatments, melatonin only

caused a shift in fungal communities at high melatonin treatments in soil W under high cadmium stress, and to low melatonin under low salt stress in soil C (Table 2.2).

Table 2.2: Responses of bacterial and fungal communities to individual treatments of melatonin under various stressor conditions as determined by PERMANOVA. For control treatments, melatonin was replaced with dilute ethanol. Significant differences ($p < 0.05$) between melatonin treatments are highlighted in blue (cadmium stress) or yellow/orange (salt stress) under the following significance levels: ☐ $p > 0.05$ (n.s.); ☐: $0.01 < p \leq 0.05$; ☐: $0.001 < p \leq 0.01$; ☐: $p \leq 0.001$.

		Cadmium stress									**Salt stress**								
		Soil P			**Soil C**			**Soil W**			**Soil P**			**Soil C**			**Soil W**		
		H	**L**	**Z**	**H**	**L**	**Z**	**H**	**L**	**Z**	**H**	**L**	**Z**	**H**	**L**	**Z**	**H**	**L**	**Z**
Bacteria	High MT vs Low MT																		
	High MT vs No MT																		
	Low MT vs No MT																		
Fungi	High MT vs Low MT																		
	High MT vs No MT																		
	Low MT vs No MT																		

MT: melatonin; H: High stressor; L: Low stressor; Z: No stressor. All treatments and controls for the same soil were composed of a standardised amount of dilute ethanol. n=4 replicates per treatment. Statistical values presented in Supplementary Table 2.7.8.

2.4.2 Microbial responses within soil W communities

Only one of the six soil-stressor combinations (i.e. soil W under salt stress) showed significant ($p < 0.01$) responses to treatments of melatonin, stressor and melatonin x stressor for both fungal and bacterial communities as described by PERMANOVA (Table 2.1; Supplementary Tables 2.7.6 – 2.7.8). These communities were subsequently assessed in greater detail.

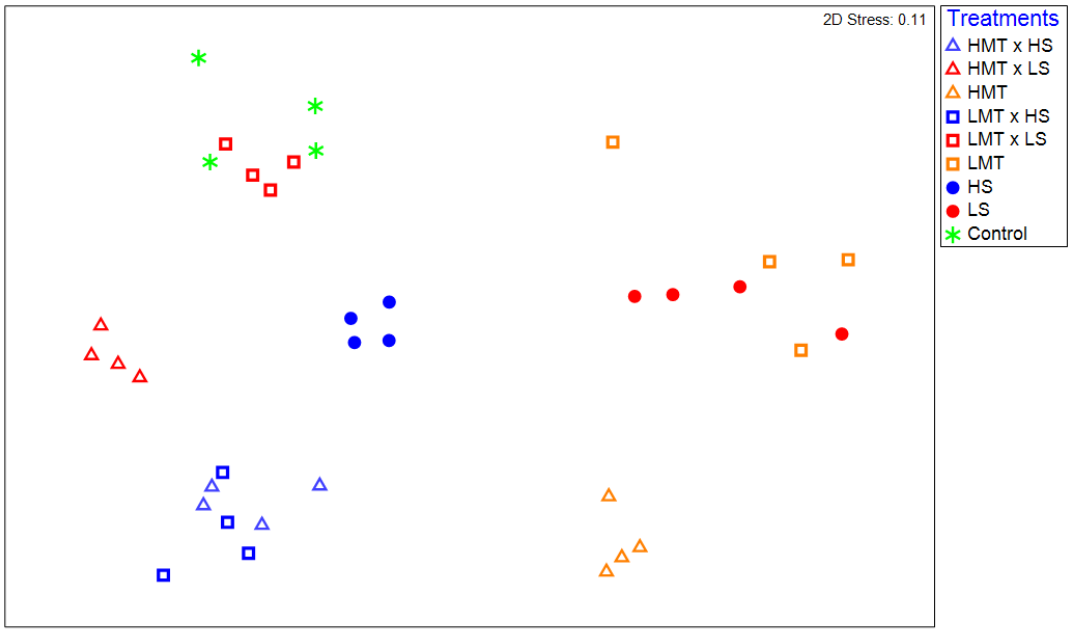


Figure 2.2: Non-metric multidimensional scaling (nMDS) ordination displaying Bray-Curtis similarities for bacterial samples within soil W for various treatments of melatonin and salt based upon community compositions determined by ARISA fingerprinting analysis. Low 2D stress value (< 0.20) indicate high quality ordination plots. Relative proximity of replicates reflects high community similarity within the same treatments for bacterial communities. Water replaced salt treatment and dilute ethanol replaced melatonin treatments in respective control samples. All treatments and controls were composed of a standardised amount of dilute ethanol. (HMT: High melatonin; LMT: Low melatonin; HS: High salt; LS: Low salt; HMT x HS: High melatonin with high salt; HMT x LS: High melatonin with low salt; LMT x HS: low melatonin with high salt; LMT x LS: Low melatonin with low salt).

2.4.2.1 Bacterial communities show distinct responses to melatonin under salt stress in soil W

The nMDS plot for bacterial assemblages showed clear separation of samples according to treatments, with both melatonin and salt treatments resulting in shifts in community structures compared to the control (Figure 2.2). Low 2D stress values (< 0.20) indicated high quality ordination plots. Various individual OTUs shifted in response to the different treatments (Figure 2.3, Supplementary Figure 2.7.6a). Bacterial communities treated with melatonin were significantly (PERMANOVA $F_{(2, 27)} = 7.839$, $p < 0.001$) different to control communities, with high melatonin concentration being 50.5% dissimilar to the control treatments whereas low melatonin concentration samples

showed 48.0% dissimilarity (Supplementary Table 2.7.9a). Similarity Percentage Analysis (SIMPER) showed the OTUs contributing most to assemblage differences upon high melatonin treatment in comparison to control samples. This analysis found that the top three OTUs accounted for 27% of the total dissimilarity. These were OTU: 849 bp (12.9%), 741 bp (7.3%) and 756 bp (6.8%). High and low concentration melatonin samples were 36.7% dissimilar to each other with 29 OTUs accounting for 71.1% of these differences, the highest of which (OTU: 180 bp) represented only 5.0% of the dissimilarity (Supplementary Figure 2.7.6a). Bacterial responses to both concentrations of melatonin for soil W were significant under high and low salt stress in comparison to the respective salt only treatments (Table 2.2).

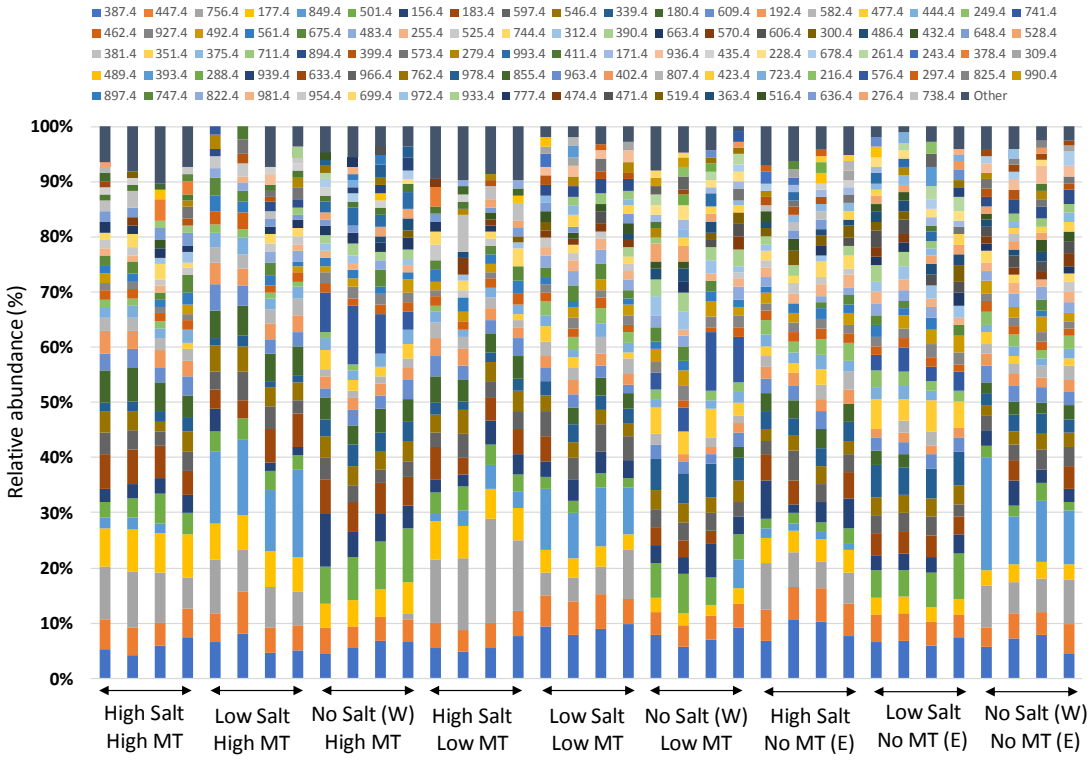


Figure 2.3: Relative abundances of individual OTUs in bacterial samples associated with soil W for various treatments of melatonin and salt based upon community compositions determined by ARISA fingerprinting analysis. Legends represent different individual OTUs as determined by specified nucleotide lengths. Water (W) replaced salt treatment and dilute ethanol (E) replaced melatonin treatments in respective control samples. All treatments and controls were composed of a standardised amount of dilute ethanol. MT: Melatonin.

Under all the above comparisons, a single OTU (756 bp) consistently increased (up to 7.7%) when melatonin was present, independent of melatonin and salt concentrations. Interestingly, some OTUs most responsive to melatonin under high salt conditions in soil W (e.g. OTU 387 bp) were far less responsive to melatonin under low salt conditions and vice versa (e.g. OTU 849 bp).

2.4.2.2 Fungal community responses to treatments (melatonin and/or salt) in soil W

NMDS ordination for fungal communities within soil W indicated significant separation of melatonin (PERMANOVA: $F_{(2,\ 27)} = 2.131$, $p < 0.001$) and salt (PERMANOVA: $F_{(2,\ 27)} = 6.027$, $p < 0.001$) treated samples with respective control samples (Figure 2.4 & 2.5).

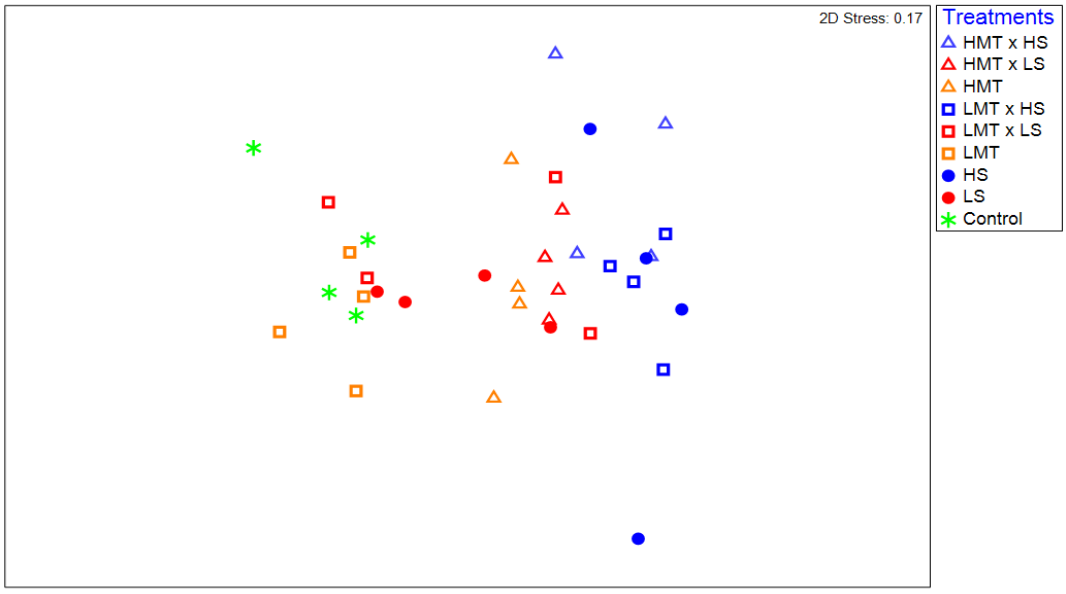


Figure 2.4: Non-metric multidimensional scaling (nMDS) ordination displaying Bray-Curtis similarities for fungal samples within soil W for various treatments of melatonin and salt based upon community compositions determined by ARISA fingerprinting analysis. Low 2D stress value (< 0.20) indicate high quality ordination plots. Relative proximity of replicates reflects high community similarity within the same treatments for bacterial communities. Water replaced salt treatment and dilute ethanol replaced melatonin treatments in respective control samples. All treatments and controls were composed of a standardised amount of dilute ethanol. (HMT: High melatonin; LMT: Low melatonin; HS: High salt; LS: Low salt; HMT x HS: High melatonin with high salt; HMT x LS: High melatonin with low salt; LMT x HS: low melatonin with high salt; LMT x LS: Low melatonin with low salt).

Relative abundances of individual fungal OTUs varied across all treatments in soil W under salt stress (Supplementary Figure 2.7.6b). Fungal communities in soil W treated with high melatonin concentration showed greater dissimilarity (33.7%) than low melatonin-treated samples (28.5%) when both were compared with control samples (Supplementary Table 2.7.9b), with high melatonin treatment significantly different ($p < 0.05$) to control samples (Table 2.2).

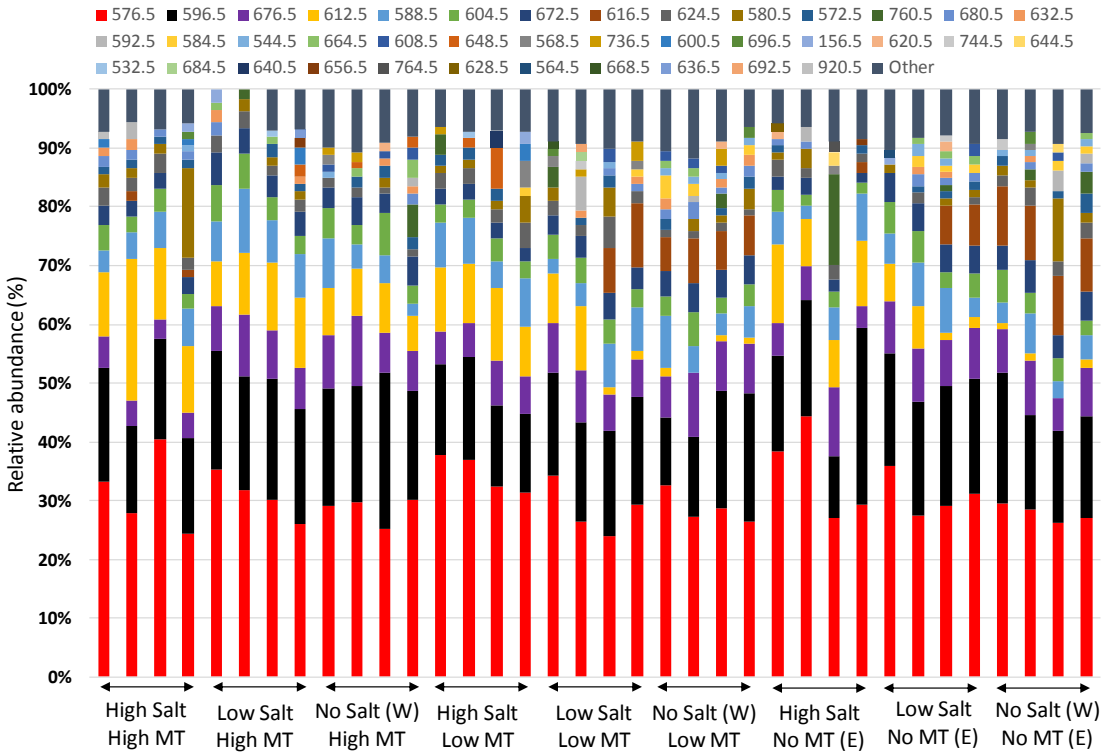


Figure 2.5: Relative abundances of individual OTUs in fungal samples associated with soil W for various treatments of melatonin and salt based upon community compositions determined by ARISA fingerprinting analysis. Legends represent different individual OTUs as determined by specified nucleotide lengths. Water (W) replaced salt treatment and dilute ethanol (E) replaced melatonin treatments in respective control samples. All treatments and controls were composed of a standardised amount of dilute ethanol. MT: Melatonin.

In soil W communities treated with high melatonin concentration, three taxa accounted for almost half of the total dissimilarity: OTUs 607 bp (18.6%), 575 bp (16.8%) and 591 bp (13.2%). Correspondingly, OTUs 575 bp (19.2%) and 591 bp (13.8%) also accounted strongly for dissimilarity between control and low melatonin-treated samples (Supplementary Figure 2.7.6b). Separation between samples treated with low melatonin

concentration only compared with samples treated with both salt and low melatonin concentration was observed; however fungal community differences were not determined as significant in response to melatonin under salt stress (Figure 2.4; Table 2.2).

2.5 Discussion

Soil microbial communities are a key component of a healthy ecosystem, providing several ecosystem services including direct and indirect nourishments of plant root systems (Wall and Virginia, 1999; Yao *et al.*, 2000; Kirk *et al.*, 2004; Wahid *et al.*, 2016). Significant responses of microbes at a community level to melatonin under normal or abiotic stress conditions in agricultural top-soils (10cm depth) were detected. Shifts in soil microbial communities may result in changes to various ecosystem services provided by soil microbes (Mau *et al.*, 2019; Zhang *et al.*, 2019). The data in the current study therefore suggests that any potential applications of melatonin in future agricultural practices must also investigate the resulting shift to soil microbial communities to ensure that plant-soil ecology interactions are not negatively impinged in the long term.

It was hypothesised that melatonin would alter soil microbial community structures, as previous reports suggest that melatonin may act with antimicrobial properties. For example, melatonin was shown to inhibit the *in vitro* growth of the human bacterial pathogens, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* at concentrations between 130-530 µM (Tekbas *et al.*, 2008). Melatonin has also been reported to inhibit the *in vitro* growth of the human pathogenic yeast, *Candida albicans*, albeit at a much higher concentration (1300 µM) (Öztürk *et al.*, 2000). In the current study, bacterial communities in three different agricultural soils were affected by melatonin alone, however, fungal communities were less responsive to melatonin compared with bacteria (Table 2.2; Supplementary Table 2.7.8). *In vitro* studies investigating responses of filamentous fungi to melatonin complement these finding as melatonin, at very high concentration (100 mM), showed no impact on the *in-vitro* growth of *Physalospora piricola*, *Botrytis cinerea* or *Mycosphaerella arachidicola* (Wang *et al.*, 2001). Growth of *Alternaria* spp. has been reported to be inhibited at a 4 mM (Arnao and Hernández-Ruiz, 2015). Taken together the results in the current study from soil bacteria analysis indicate a similar trend compared to the *in vitro* experiments albeit different melatonin concentration ranges.

Recently Li *et al.* investigated the effect of exogenous melatonin application (200 µM; applied at 20 day intervals for 6 months) without abiotic stress in two soils types associated with horticultural practices (apple orchard and vegetables respectively) by sampling the subsoil region (20-30 cm depth) (Li *et al.*, 2018). Bacterial compositions of melatonin-treated soil samples were shown to be similar to controls, however some genera, many unknown, shifted strongly in response to melatonin (Li *et al.*, 2018). Ascomycota, in particular, were negatively affected by melatonin, resulting in greater establishment of *Glomeromycota* and *Basidiomycota* in fungal assemblages (Li *et al.*, 2018). It is difficult to directly compare the current study with this report; however, these results are complimentary to the trends observed in my investigation and indicative of the importance of soil agricultural histories in microbial response to melatonin. In the current study, topsoils were used, which are generally more microbially rich (compared to subsoils), as well as soils associated with agricultural practices (crop production and pasture). Further analysis by taxa identification of the soil microbial communities associated with responses to melatonin would determine those microbes associated with particular ecological niches (e.g. mycorrhizal fungi or Plant Growth-Promoting Rhizobacteria) and if they are known to be beneficial to crops.

Under abiotic stresses, such as cadmium and salt, plants can cope better by adjusting physiological and enzymatic processes when melatonin is applied (Li *et al.*, 2012; Byeon *et al.*, 2015; Liang *et al.*, 2015; Jiang *et al.*, 2016; Li *et al.*, 2016; Gu *et al.*, 2017). Some reports also suggested that melatonin may enhance abiotic stress tolerance in microbes as endogenous levels of melatonin increased in *Trichoderma* spp. (Liu *et al.*, 2016) and *Saccharomyces cerevisiae* (Rodriguez-Naranjo *et al.*, 2012) under abiotic stresses (cadmium and ethanol respectively). Cadmium chloride hemipentahydrate (280 or 100 mg kg^{-1} soil) and salt (NaCl) (7 or 4 g kg^{-1} soil) were separately applied as abiotic stressors in the current study. These concentrations were selected to be within the range of those reported to induce effects on soil microbial activities in previous studies for cadmium (Cáliz *et al.*, 2013; Wood *et al.*, 2016b) and salt (Rath *et al.*, 2016). For example, Wood *et al.* (2016b) found that soil treated with cadmium chloride ($CdCl_2$) at 100 mg kg^{-1} soil resulted in significant shifts in bacterial community structures, whereas fungal communities were unaffected at this concentration of soil contamination. Cáliz *et al.* (2013) found that significant impacts to microbial communities in soils treated with cadmium sulphate ($CdSO_4$) at 1000 mg kg^{-1} soil.

Salinity altered the bacterial and fungal community structures of root microsymbionts associated with alder (*Alnus glutinosa*) (Thiem *et al.*, 2018). Rath *et al.*, (2016) found effects on soil bacterial and fungal growth as well as microbial respiration at 63, 55 and 79 mmol $NaCl.kg^{-1}$ soil respectively. This corresponds closely to the equivalent concentrations of NaCl applied in the current experiment for 4 $mg.kg^{-1}$ (68 mmol $NaCl.kg^{-1}$ soil). The current study found that bacterial communities showed more significant responses to melatonin under abiotic stress conditions compared to fungi, along with more distinct separation of communities per treatment (e.g. Soil W). Previous soil studies have also found soil bacterial communities to be more responsive to various stress treatments in comparison to fungal communities, with fungi showing greater tolerance to abiotic stressors (Hiroki, 1992; Müller *et al.*, 2001; Marschner *et al.*, 2003; Rajapaksha *et al.*, 2004).

The different responses of soil microbes across the three agricultural soils to melatonin and/or stressors may also have been in part due to differing soil physiochemical characteristics between the soils, as well as differing interactive effects of treatments with various soil characteristics (Zhong and Cai, 2007; Ahn *et al.*, 2012; Zhao *et al.*, 2014; Geisseler *et al.*, 2017). In the current study, some communities were far less impacted by abiotic stress upon the availability of exogenous melatonin. However, as this trend was not observed under cadmium stress, it may be possible that melatonin was utilised by soil bacteria to sustain natural microbial activity by coping with impacts specific to salt stress, such as enhanced osmotic pressure and ion toxicity (Morrissey *et al.*, 2014; Yan *et al.*, 2015).

Melatonin has been consistently shown to reduce cellular levels of ROS in plants exposed to abiotic stress (Tan *et al.*, 2012), by either acting as a highly efficient antioxidant (Hardeland, 1999; Reiter *et al.*, 2015; Reiter *et al.*, 2016), or as a signalling molecule, resulting in the upregulation of gene expression, or increased enzyme activities of ROS-scavenging enzymes (Rodriguez *et al.*, 2004; Lee *et al.*, 2014; Manchester *et al.*, 2015; Zhang *et al.*, 2015). As a result, melatonin enhances plant tolerance to abiotic and biotic stresses such as heat, cold, drought and soil contamination as well bacterial and fungal pathogens (Arnao and Hernández-Ruiz, 2013; Arnao and Hernandez-Ruiz, 2014; Zhang *et al.*, 2015; Hardeland, 2016). Melatonin has also been found to increase plant yield by acting as a biostimulator for seed germination and plant growth. As melatonin is safe for human consumption and

can be applied to plants in numerous ways such as seed coating, foliar spraying or soil treatment, it may therefore have a major role in future agricultural practices for crop yield protection and improvement (Janas and Posmyk, 2013; Wei *et al.*, 2015; Cui *et al.*, 2017; Zhang *et al.*, 2017).

2.6 Conclusion

In conclusion, this study has demonstrated that exogenous melatonin altered the structures of soil bacterial and, to a lesser extent, fungal assemblages under unstressed and abiotic stressed conditions. No previous reports have examined the effects of melatonin on agricultural soil microbial communities under abiotic stress. Further research is required to profile the microbial taxa responsive to melatonin as well as investigate potential functional associations between melatonin with abiotic stress tolerance in microbes. The main factors causing the differences in natural microbial communities between the different soils also requires further analysis. Additional research is also required to determine if specific soil characteristics influence the responses of microbial communities to melatonin. Moreover, studies may explore potential plant-microbe interactions in soil upon the bioavailability of exogenous melatonin. Future studies involving ameliorating plant stress using melatonin should take into account the potential impact of soil microbiota and the subsequent impact on plant-microbe interactions (beneficial as well as pathogenic). Understanding the role of melatonin in soil microbial community dynamics may provide vital information regarding the viability of melatonin application relating to future agricultural practices.

2.7 Supplementary Information

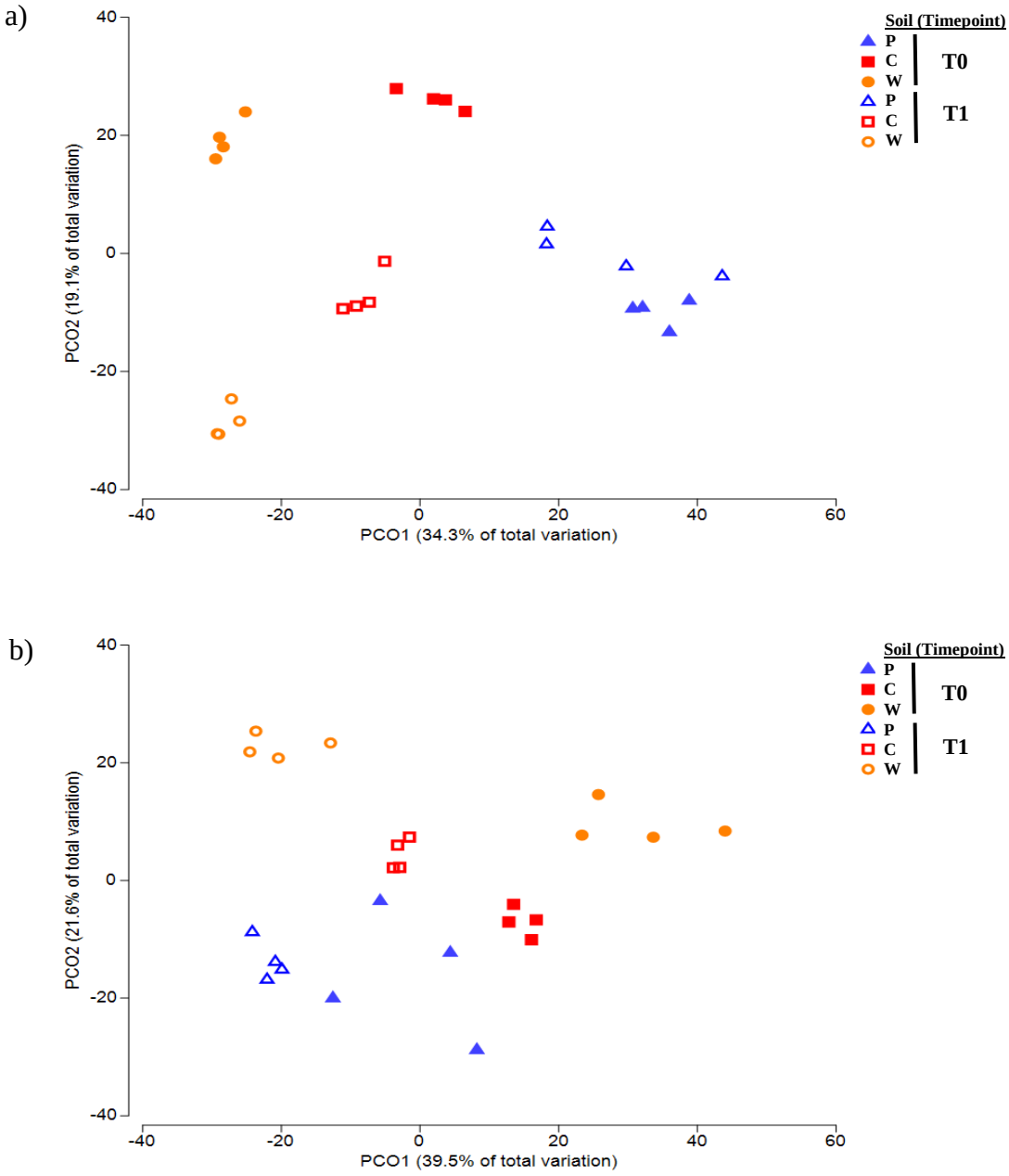
Supplementary Table 2.7.1: Soil physical and chemical characteristics of three sampled topsoils (0-10 cm) prior to treatments. Four topsoil samples were collected at each site and composited prior to analysis.

Site	**P**	**C**	**W**
Available Potassium (mg kg^{-1} soil)	640	140	170
pH (1:5 $CaCl_2$)	6.1	4.9	4.7
Organic Carbon (%)	4.6	3	2.8
Nitrate N (mg kg^{-1} soil)	160	57	34
Ammonium N (mg kg^{-1} soil)	2.5	8.9	8.8
Phosphorus (Colwell) (mg kg^{-1} soil)	160	41	32
Phosphorus Buffer Index	32	71	83
Calcium (cmol(+) kg^{-1} soil)	12	5.1	4.7
CEC (cmol(+) kg^{-1} soil)	15.7	7.94	8.56
EC (1:5 water) (dS m^{-1})	0.37	0.24	0.18
Chloride (mg kg^{-1} soil)	37	67	76
Moisture (%)	14	6	5.2
Water holding capacity (%)	38.4	24.6	21.1
Sand (%)	75.2	53.9	52.4
Silt (%)	23.6	29.9	28.8
Clay (%)	1.2	16.2	18.8
Iron (mg kg^{-1} soil)	5400	16000	21000
Zinc (mg kg^{-1} soil)	23	7.2	9.3
Cadmium (mg kg^{-1} soil)	0.24	0.13	0.2
Chromium (mg kg^{-1} soil)	11	17	32
Nickel (mg kg^{-1} soil)	4	4.7	22
Lead (mg kg^{-1} soil)	7.7	9.4	16

CEC = cation exchange capacity; EC = electrical conductivity; Soil P – recently (< 3 months) associated with cattle and sheep pasture; Soil C – sampled 3 weeks post canola harvest; Soil W –sampled 3 weeks post fire blazing following a wheat harvest.

Supplementary Table 2.7.2: Salinity levels of soils P, C and W treated with high (7 g kg^{-1} soil) and low (4 g kg^{-1} soil) Salt (NaCl) only, as measured by electrical conductivity [EC (1:5 soil:water)]. SE: Standard error

	EC 1:5 (mS/cm)								
	Soil P			**Soil C**			**Soil W**		
	7 g kg^{-1}	**4 g kg^{-1}**	**Control**	**7 g kg^{-1}**	**4 g kg^{-1}**	**Control**	**7 g kg^{-1}**	**4 g kg^{-1}**	**Control**
Rep 1	2.6	1.72	0.706	1.97	1.015	0.091	1.934	1.148	0.101
Rep 2	2.545	1.708	0.678	1.943	1.083	0.132	1.954	1.089	0.093
Rep 3	2.502	1.8	0.681	1.829	1.084	0.138	1.983	1.065	0.102
Mean	**2.549**	**1.743**	**0.688**	**1.914**	**1.061**	**0.120**	**1.957**	**1.101**	**0.099**
SE	0.028	0.029	0.009	0.043	0.023	0.015	0.014	0.025	0.003



Supplementary Figure 2.7.1: Principal coordinates analysis (PCOA) ordination plots (Bray-Curtis distance matrix) of ARISA profiles showing the separation within a) bacterial and b) fungal communities for the three dry, untreated soils (P, C, W; n = 4). Bacterial communities from the same soil differed (ANOSIM: R=0.865 -1; p = 0.029) between sampling timepoints T0 and T1 within soils C and W, whereas bacterial communities in soil A were not significantly different between timepoints T0 and T1 (ANOSIM: R=0.594; p = 0.057). All untreated soil communities from different soils were dissimilar ($p < 0.05$) to each other. Fungal communities from the same soil differed (ANOSIM: R = 0.458 – 1; p = 0.029) between T0 and T1 for all three soils.

2.7.1 Effects of treatments on microbial diversity (OTU richness)

Community fingerprinting with ARISA allowed for a statistical estimation of the α diversity based on the different Operational Taxonomic Units (OTUs) observed. The basal levels of α diversity determined in the dry, untreated samples from P, C and W indicated a mean of 29.7, 30 and 36 bacterial OTU's, and 16.6, 16.4 and 14.8 fungal OTU's, respectively. Concurrently, across all three control soil samples, the number of OTUs observed for bacterial communities (23-39) were considerably greater than the OTU numbers for fungi (8-20). Such findings are in line with other studies where community fingerprinting with ARISA detected substantially fewer fungal OTUs compared to bacterial (Ranjard *et al.*, 2001; Hansgate *et al.*, 2005).

2.7.1.1 Melatonin alters microbial α diversity

In melatonin-treated soils the mean bacterial OTU richness (α diversity) was 29.9, 34.3 and 34.2 in soils P, C and W (ranging from 21 to 40 OTU's; Supplementary Table 2.7.3a) and 16.1, 14.4 and 13 (ranging from 9 to 19 OTU's; Supplementary Table 2.7.3b) for fungi respectively. The effect of melatonin treatment at high (H) and low (L) concentrations on OTU numbers varied for bacterial communities across the three soils compared to control samples (Supplementary Table 2.7.3a). Bacterial α diversities shifted ($p < 0.05$) in response to high melatonin treatment within all three soils, whereas low melatonin treatment impacted bacterial community only in soil C. At sampling timepoints T0 and T1, bacterial OTU richness's were significantly ($p < 0.05$) decreased compared to respective control samples upon treatment with high melatonin for soil W. High melatonin also resulted in significant decrease in bacterial OTU numbers in soil P relative to controls at T0, but not T1. Interestingly bacterial OTU richness in high and low melatonin-treated soils for soil C significantly ($p < 0.01$) increased at sampling timepoint T1, but no significant changes were observed at T0. In contrast to the varying responses of bacterial assemblages, OTU richness of fungal communities across all three soils was not impacted ($p > 0.05$) by melatonin (Supplementary Table 2.7.3a).

The number of bacterial OTUs common to control and melatonin-only treatments varied considerably for each soil between the sampling timepoints T0 and T1. For example, at T0, 73.1% (49/67), 73.7% (45/61) and 73.2% (52/71) of bacterial OTUs were common to control and melatonin-only treatments in soils P, C and W respectively. However, these numbers reduced at T1 to 56.4% (44/78), 43.6% (34/78) and 55.3% (42/76) respectively. Fungal OTUs common to control and melatonin-only

treatments in soils varied less compared to bacterial assemblages. At timepoint T0, 75.8% (22/29), 71.4% (20/28) and 76.2% (16/21) fungal OTUs were common to control and melatonin-only treatments in soils P, C and W respectively (Supplementary Table 2.7.3b). At T1, the common OTUs reduced to 63.6% (21/33) for soil P, whereas OTU richness increased slightly to 74.1% (20/27) and 80.8% (21/26) for soils C and W respectively.

2.7.1.2 Cadmium and salt show limited impacts on microbial α diversity

OTU richness (α diversity) was not impacted by stressor level (H or L) in cadmium or salt experiment (PERMANOVA, $p > 0.05$) for bacterial communities in soils P and W (Supplementary Table 2.7.3). For soil C, low salt treatment significantly increases in OTU numbers ($p < 0.001$) relative to control. OTU numbers for fungal communities across all soils were not impacted ($p > 0.05$) by either cadmium or salt stresses.

The number of bacterial OTUs common to cadmium-only treatments and the control samples for each soil were relatively consistent within soils P, C and W at 63.0% (46/73), 60.3% (41/68) and 59.5% (44/74) respectively. In contrast, bacterial OTU numbers common to salt-only and control treatments showed greater variation at 67.6% (48/71), 45.3% (34/75) and 60.8% (45/74) for soils P, C and W respectively. Fungal OTUs common to cadmium-only treatments and the control samples were varied for soils P, C and W at 73.3% (22/30), 68.0% (17/25) and 84.2% (16/19) respectively. In comparison, fungal OTUs for salt-only and control treatments showed greater consistency with 75.8% (22/29), 71.4% (20/28) and 75.8% (22/29) common across soils P, C and W respectively.

Supplementary Table 2.7.3: Mean OTU richness (α diversity) (± SE) within a) bacterial and b) fungal communities for various melatonin- or stressor-only treatments (n=4 replicates). MT = Melatonin (High concentration = 4 mg kg^{-1} soil; Low concentration = 0.2 mg kg^{-1} soil). Stressors: T0 = cadmium (High concentration = 280 mg kg^{-1} soil; Low concentration = 100 mg kg^{-1} soil); T1 = salt (High concentration = 7 g kg^{-1} soil; Low concentration = 4 g kg^{-1} soil). Melatonin-treated soils contained no abiotic stressor. Control = dilute ethanol solution (i.e. no melatonin, no stressor).

a)

	Soil P		**Soil C**		**Soil W**	
	T0	**T1**	**T0**	**T1**	**T0**	**T1**
High MT	**24.0 ± 1.5** *	33.5 ± 2.3	33.8 ± 1.7	**36.3 ± 0.9** *	**28.8 ± 1.0** *	**34.3 ± 0.3** *
Low MT	28.8 ± 1.1	33.5 ± 2.8	30.8 ± 2.8	**36.5 ± 0.9** *	37.5 ± 0.9	36.3 ± 1.0
High stress	31.0 ± 0.4	33.8 ± 0.8	28.3 ± 1.9	22.5 ± 0.6	34.0 ± 0.4	37.5 ± 0.5
Low stress	31.3 ± 0.8	32.3 ± 0.8	28.8 ± 1.1	**36.5 ± 1.3** *	34.3 ± 0.5	37.5 ± 0.6
Control	31.5 ± 1.0	31.8 ± 0.8	33.8 ± 1.7	26.0 ± 1.1	37.0 ± 0.4	38.3 ± 0.8

b)

	Soil P		**Soil C**		**Soil W**	
	T0	**T1**	**T0**	**T1**	**T0**	**T1**
High MT	17.3 ± 1.0	14.5 ± 1.0	16.8 ± 0.8	15.0 ± 0.7	13.3 ± 0.9	11.0 ± 1.1
Low MT	16.8 ± 0.5	15.8 ± 1.5	12.8 ± 1.3	13.3 ± 0.9	16.3 ± 0.6	11.8 ± 0.5
High stress	15.8 ± 1.7	17.3 ± 0.5	16.0 ± 1.4	13.5 ± 0.5	11.8 ± 0.5	12.3 ± 0.5
Low stress	18.0 ± 1.1	15.8 ± 0.6	15.0 ± 1.2	14.3 ± 0.5	14.3 ± 1.7	11.5 ± 0.3
Control	17.8 ± 1.1	16.0 ± 1.2	16.0 ± 0.9	13.0 ± 0.6	15.0 ± 0.9	10.8 ± 1.1

All treatments and controls in the same soil were composed of a standardised amount of dilute ethanol. OTU numbers in bold were determined as being significantly different to control samples using one-way ANOVA. * indicates significance level of 0.001.

2.7.2 Bacterial and fungal OTUs most responsive to melatonin

Supplementary Table 2.7.4: The largest increases of relative abundance (%) of individual OTUs from melatonin-only treated samples compared with controls as determined by ANOSIM.

Bacteria

Timepoint	MT treatment	Soil	OTU	MT abundance (%)	Control Abundance (%)	Difference (%)
T1	High	W	741.4	7.09	0	7.1
T1	Low	W	741.4	6.55	0	6.6
T0	High	P	447	16.98	10.44	6.5
T0	High	W	447	10.54	4.13	6.4
T1	Low	C	741.4	8.34	2.21	6.1
T1	High	W	501.4	8.22	2.2	6.0
T1	High	C	741.4	7.31	2.21	5.1
T0	High	W	183	9.12	4.36	4.8
T1	High	C	447.4	8.64	4.27	4.4
T1	Low	C	384.4	4.27	0	4.3
T1	Low	C	501.4	6.92	2.81	4.1
T1	High	W	183.4	5.58	1.9	3.7
T1	Low	W	501.4	5.75	2.2	3.6

Fungi

Timepoint	MT treatment	Soil	OTU	MT abundance (%)	Control Abundance (%)	Difference (%)
T0	High	W	607.5	17.33	5.8	11.5
T1	High	W	612.5	7.63	0.92	6.7
T0	Low	C	599.5	7.24	1.28	6.0
T0	High	W	675.5	9.55	4.23	5.3
T0	Low	P	567.5	11.79	7.21	4.6
T0	High	C	575.5	45.02	40.96	4.1
T0	High	P	559.5	7.89	3.95	3.9
T0	Low	C	591.5	11.69	8.11	3.6
T1	High	W	596.5	21.24	17.84	3.4
T0	Low	W	607.5	8.96	5.8	3.2
T1	High	P	676.5	5.19	2.13	3.1
T1	Low	C	576.5	37.42	34.42	3.0
T1	Low	P	708.5	2.74	0	2.7

MT – melatonin; OTU: Operational Taxonomic Unit; Soil P: Pasture; Soil C: Canola; Soil W: Wheat [+fireblazing]; High MT = 4 mg kg^{-1} soil; Low MT = 0.2 mg kg^{-1} soil.

Supplementary Table 2.7.5: The largest decreases of relative abundance (%) of individual OTUs from melatonin-only treated samples compared with controls as determined by ANOSIM.

Bacteria

Timepoint	MT treatment	Soil	OTU	MT abundance (%)	Control Abundance (%)	Difference (%)
T1	High	C	849.4	2.42	19.34	-16.9
T1	Low	C	756.4	2.69	18.89	-16.2
T1	High	C	756.4	2.79	18.89	-16.1
T1	Low	C	849.4	3.3	19.34	-16.0
T1	High	W	849.4	0	12.48	-12.5
T1	Low	W	849.4	1.28	12.48	-11.2
T1	Low	W	756.4	0	6.9	-6.9
T1	High	W	756.4	0.28	6.9	-6.6
T0	High	W	339	3.06	8.56	-5.5
T1	Low	P	156.4	2.33	6.43	-4.1
T0	Low	C	738	4.61	8.36	-3.8
T1	Low	P	180.4	2.27	5.86	-3.6
T1	High	P	180.4	2.74	5.86	-3.1

Fungi

Timepoint	MT treatment	Soil	OTU	MT abundance (%)	Control Abundance (%)	Difference (%)
T0	Low	C	575.5	30.73	40.96	-10.2
T1	High	W	616.5	0	9.6	-9.6
T0	High	W	591.5	15.82	23.59	-7.8
T0	Low	W	591.5	17.63	23.59	-6.0
T0	High	W	755.5	0.38	6.29	-5.9
T0	High	W	575.5	27.67	33.45	-5.8
T0	Low	W	755.5	1.8	6.29	-4.5
T1	High	P	576.5	31.3	35.6	-4.3
T1	High	P	876.5	0	4.24	-4.2
T1	Low	P	876.5	0	4.24	-4.2
T0	High	C	607.5	6.98	11.13	-4.2
T0	High	P	591.5	12.71	16.72	-4.0
T1	Low	C	584.5	1.05	4.72	-3.7

MT – melatonin; OTU: Operational Taxonomic Unit; Soil P: Pasture; Soil C: Canola; Soil W: Wheat [+fireblazing]; High MT = 4 mg kg^{-1} soil; Low MT = 0.2 mg kg^{-1} soil.

2.7.3 Effects of treatments on microbial community structures (β diversity)

Supplementary Table 2.7.6: PERMANOVA analyses of a) bacterial and b) fungal community responses to all treatments with high and low melatonin (i.e. no stressors included) at sampling timepoints T0 and T1, based on ARISA data (Bray-Curtis dissimilarity distances).

a)

	Soil P			
	T0		**T1**	
	t-statistic	**P-value**	**t-statistic**	**P-value**
H-MT vs L-MT	0.98661	0.4074	1.5173	0.0103*
H-MT vs Control	1.3386	0.0928	1.7634	0.0008***
L-MT vs Control	1.2234	0.1587	1.3574	0.0665

	Soil C			
	T0		**T1**	
	t-statistic	**P-value**	**t-statistic**	**P-value**
H-MT vs L-MT	1.6225	0.0263*	1.1227	0.2484
H-MT vs Control	0.8002	0.7636	1.8497	0.0101*
L-MT vs Control	1.7851	0.0135*	2.2132	0.0029**

	Soil W			
	T0		**T1**	
	t-statistic	**P-value**	**t-statistic**	**P-value**
H-MT vs L-MT	3.5945	0.0001***	2.8317	0.0001***
H-MT vs Control	4.3751	0.0001***	3.7099	0.0001***
L-MT vs Control	1.3464	0.0261*	1.6455	0.0006***

b)

	Soil P			
	T0		T1	
	t-statistic	P-value	t-statistic	P-value
H-MT vs L-MT	1.2699	0.1042	1.0662	0.3265
H-MT vs Control	0.92172	0.5693	1.4434	0.0194*
L-MT vs Control	1.0088	0.4313	1.0374	0.3796

	Soil C			
	T0		T1	
	t-statistic	P-value	t-statistic	P-value
H-MT vs L-MT	0.90452	0.6202	0.71054	0.9021
H-MT vs Control	1.1021	0.2829	1.1387	0.2269
L-MT vs Control	1.063	0.3425	1.304	0.0727

	Soil W			
	T0		T1	
	t-statistic	P-value	t-statistic	P-value
H-MT vs L-MT	1.87	0.0068**	1.7212	0.0041**
H-MT vs Control	2.7675	0.0005***	1.7703	0.0005***
L-MT vs Control	1.6514	0.0248*	0.73838	0.8333

H-MT: High melatonin; L-MT: Low melatonin. Control treatments consisted of dilute ethanol replacing melatonin. All treatments and controls for the same soil were composed of a standardised amount of dilute ethanol. n=4 replicates per treatment. Significance of PERMANOVA: *: $0.01 < \text{p-value} \leq 0.05$; **: $0.001 < \text{p-value} \leq 0.01$; ***: $\text{p-value} \leq 0.001$.

Supplementary Table 2.7.7: PERMANOVA analyses of a) bacterial and b) fungal community responses to treatments with high and low stresses based on ARISA data (Bray-Curtis dissimilarity distances).

a)

	Soil P			
	Cadmium		**Salt**	
	t-statistic	**P-value**	**t-statistic**	**P-value**
H-stress vs L-stress	1.0771	0.2882	2.2813	0.0003***
H-stress vs Control	1.4669	0.073	1.3298	0.0431*
L-stress vs Control	1.1248	0.2325	1.6868	0.0089**

	Soil C			
	Cadmium		**Salt**	
	t-statistic	**P-value**	**t-statistic**	**P-value**
H-stress vs L-stress	2.7886	0.0001***	5.0877	0.0001***
H-stress vs Control	4.0829	0.0001***	5.861	0.0001***
L-stress vs Control	1.933	0.0035**	1.684	0.0158*

	Soil W			
	Cadmium		**Salt**	
	t-statistic	**P-value**	**t-statistic**	**P-value**
H-stress vs L-stress	2.3933	0.0001***	3.1672	0.0001***
H-stress vs Control	3.9566	0.0001***	3.9779	0.0001***
L-stress vs Control	2.2386	0.0001***	2.3602	0.0001***

b)

	Soil P			
	Cadmium		Salt	
	t-statistic	P-value	t-statistic	P-value
H-stress vs L-stress	1.026	0.3837	1.7222	0.0031**
H-stress vs Control	1.8378	0.003**	1.3462	0.036*
L-stress vs Control	2.0151	0.0008***	1.059	0.3396

	Soil C			
	Cadmium		Salt	
	t-statistic	P-value	t-statistic	P-value
H-stress vs L-stress	1.7138	0.0076**	1.4761	0.0255*
H-stress vs Control	1.5488	0.0145*	2.8112	0.0002***
L-stress vs Control	1.1351	0.23	1.8004	0.0009***

	Soil W			
	Cadmium		Salt	
	t-statistic	P-value	t-statistic	P-value
H-stress vs L-stress	1.6613	0.0255*	2.0967	0.0002***
H-stress vs Control	1.5842	0.0103*	3.2649	0.0001***
L-stress vs Control	0.99408	0.4411	1.6346	0.0131*

Treatments: H-stress = High stressor; L-stress = Low stressor; Control = MQ water replacing stressor. All treatments and controls for the same soil were composed of a standardised amount of dilute ethanol. n=4 replicates per treatment. Significance of PERMANOVA: *: $0.01 < \text{p-value} \leq 0.05$; **: $0.001 < \text{p-value} \leq 0.01$; ***: $\text{p-value} \leq 0.001$.

Supplementary Table 2.7.8: Differences (a < 0.05) between a) bacterial and b) fungal communities treated with melatonin under various stressor conditions as determined by PERMANOVA using Monte Carlo simulation [P-(MC)]. For control treatments, melatonin was replaced with dilute ethanol.

a)

	Cadmium stress						**Salt stress**					
	High stressor		**Low stressor**		**No stressor**		**High stressor**		**Low stressor**		**No stressor**	
	t-statistic	**P-(MC)**	**t-statistic**	**P-(MC)**	**t-statistic**	**P-(MC)**	**t-statistic**	**P-(MC)**	**t-statistic**	**P-(MC)**	**t-statistic**	**P-(MC)**
Soil P												
H-MT vs L-MT	1.8896	**0.0381***	1.7726	**0.0426***	0.79519	0.5926	1.2743	0.1795	1.0879	0.3402	1.228	0.23
H-MT vs Control	2.0701	**0.0174***	1.0604	0.3504	1.044	0.3492	1.8399	**0.0221***	1.6486	0.0723	0.9332	0.4988
L-MT vs Control	1.4365	0.1102	1.3795	0.135	1.0178	0.3787	1.3481	0.1393	1.2687	0.2054	1.3996	0.1239
Soil C												
H-MT vs L-MT	0.67148	0.7288	1.2021	0.241	1.5997	0.0869	0.9219	0.4359	1.8272	**0.0367***	1.1973	0.2436
H-MT vs Control	1.3559	0.1542	0.85514	0.5686	0.6089	0.8136	0.92863	0.4766	3.2053	**0.0021****	3.9213	**0.0006*****
L-MT vs Control	1.5054	0.0911	0.69004	0.7426	1.6304	0.0763	1.2148	0.2422	2.527	**0.0072****	4.1967	**0.0006*****
Soil W												
H-MT vs L-MT	1.4721	0.1032	2.502	**0.0056****	3.2428	**0.0017****	1.3523	0.1416	2.7151	**0.0039****	2.2224	**0.0106***
H-MT vs Control	2.7945	**0.0041****	2.4253	**0.0071****	3.0177	**0.0031****	2.2808	**0.0068****	4.4083	**0.0004*****	4.3159	**0.0005*****
L-MT vs Control	1.9951	**0.0123***	1.1457	0.2718	0.74773	0.7544	2.4454	**0.0052****	3.6857	**0.0013****	3.1744	**0.0031****

b)

	Cadmium stress						Salt stress					
	High stressor		Low stressor		No stressor		High stressor		Low stressor		No stressor	
	t-statistic	P-(MC)	t-statistic	P-(MC)	t-statistic	P-(MC)	t-statistic	P-(MC)	t-statistic	P-(MC)	t-statistic	P-(MC)
Soil P												
H-MT vs L-MT	1.1042	0.3099	1.3741	0.1364	1.1155	0.2973	1.2556	0.1849	0.83979	0.616	1.2733	0.1992
H-MT vs Control	1.2813	0.2003	0.764	0.6662	1.0154	0.4022	1.4583	0.0879	0.91786	0.5061	1.2197	0.2311
L-MT vs Control	0.99918	0.4228	1.1821	0.2647	0.64845	0.8226	1.2441	0.1989	0.97139	0.4403	1.1837	0.2552
Soil C												
H-MT vs L-MT	0.60558	0.8292	0.74193	0.7091	1.445	0.1233	0.90493	0.5295	1.0344	0.3864	0.9756	0.4543
H-MT vs Control	1.5469	0.1021	1.2024	0.2336	0.86243	0.5446	0.88789	0.5383	1.3229	0.165	1.1624	0.2566
L-MT vs Control	1.0831	0.3323	0.87236	0.5665	1.1843	0.2592	0.92217	0.511	1.7997	**0.0347***	1.2259	0.2114
Soil W												
H-MT vs L-MT	1.3697	0.1819	1.2114	0.2477	1.5938	0.0713	1.3287	0.159	1.1572	0.2749	2.1516	**0.0117***
H-MT vs Control	2.5237	**0.0085****	1.7198	0.0757	1.5971	0.0879	0.9977	0.4244	1.5354	0.0969	2.3936	**0.0092****
L-MT vs Control	2.0116	**0.0277***	1.0119	0.3928	1.042	0.3649	0.8445	0.5893	0.96995	0.4321	1.221	0.2209

H-MT: High melatonin; L-MT: Low melatonin. All treatments and controls for the same soil were composed of a standardised amount of dilute ethanol. n=4 replicates per treatment. Significance of PERMANOVA: *: $0.01 < \text{p-(MC)} \leq 0.05$; **: $0.001 < \text{p-(MC)} \leq 0.01$; ***: $\text{p-(MC)} \leq 0.001$.

2.7.4 Melatonin reduced bacterial Shannon species diversity (H') and enhanced fungal Shannon species diversity under low abiotic stress conditions

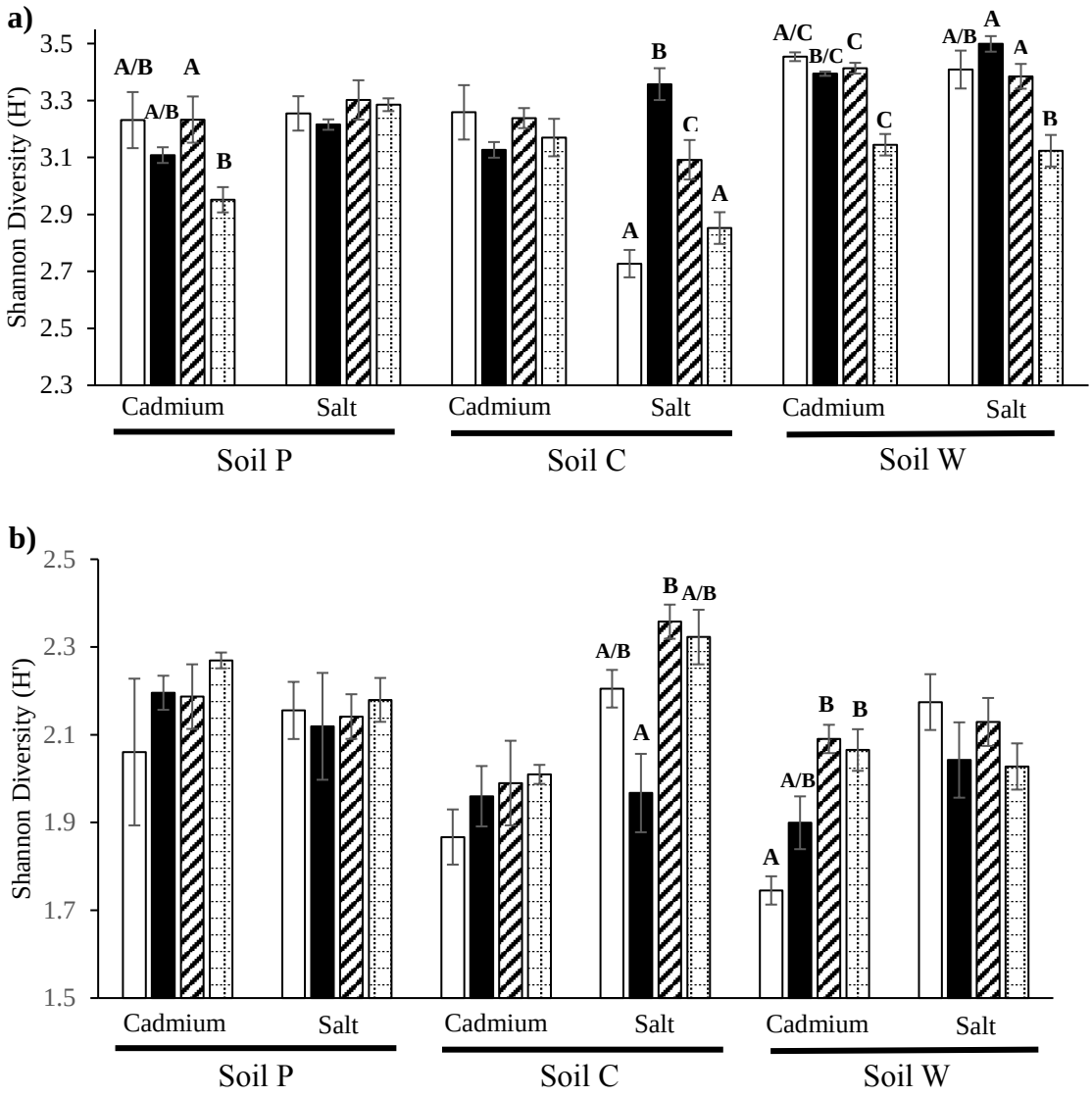
Shannon species diversity indices (H') calculated the abundance and evenness of OTUs across all soils for bacterial and fungal samples. Non-parametric pairwise Wilcoxon tests were conducted for samples within each soil and stressor treatment to determine significant differences in diversity upon the availability of melatonin. All treatments also contained a standardised dilute concentration of ethanol (approx. 0.05% v/v). Community responses to melatonin under low stress (Supplementary Figure 2.7.2) and high stress (Supplementary Figure 2.7.3) conditions were analysed.

The average Shannon's diversity index ranged from 2.73 to 3.50 for bacteria and 1.75 to 2.35 for fungi. Bacterial OTU Shannon diversity indices (H') were similar ($p > 0.05$) between control samples (0.05% v/v EtOH) and low stressor only treatments for five of the six experiments, with only low salt stress in soil C resulting in significant differences in bacterial diversity under this comparison ($p < 0.05$) (Supplementary Figure 2.7.2a). Diversity decreased in all bacterial communities impacted by melatonin under low stress conditions. Relative to the low stressor treatment within each soil, high melatonin resulted in significant decreases ($p < 0.05$) in bacterial diversity under low cadmium or salt conditions in soil W, as well as in soil C under low salt stress only. In contrast, low melatonin only resulted in a diversity shift ($p < 0.05$) in one soil treatment relative to the low stressor treatment - low salt stress in soil C ($p < 0.05$) (Supplementary Figure 2.7.2a).

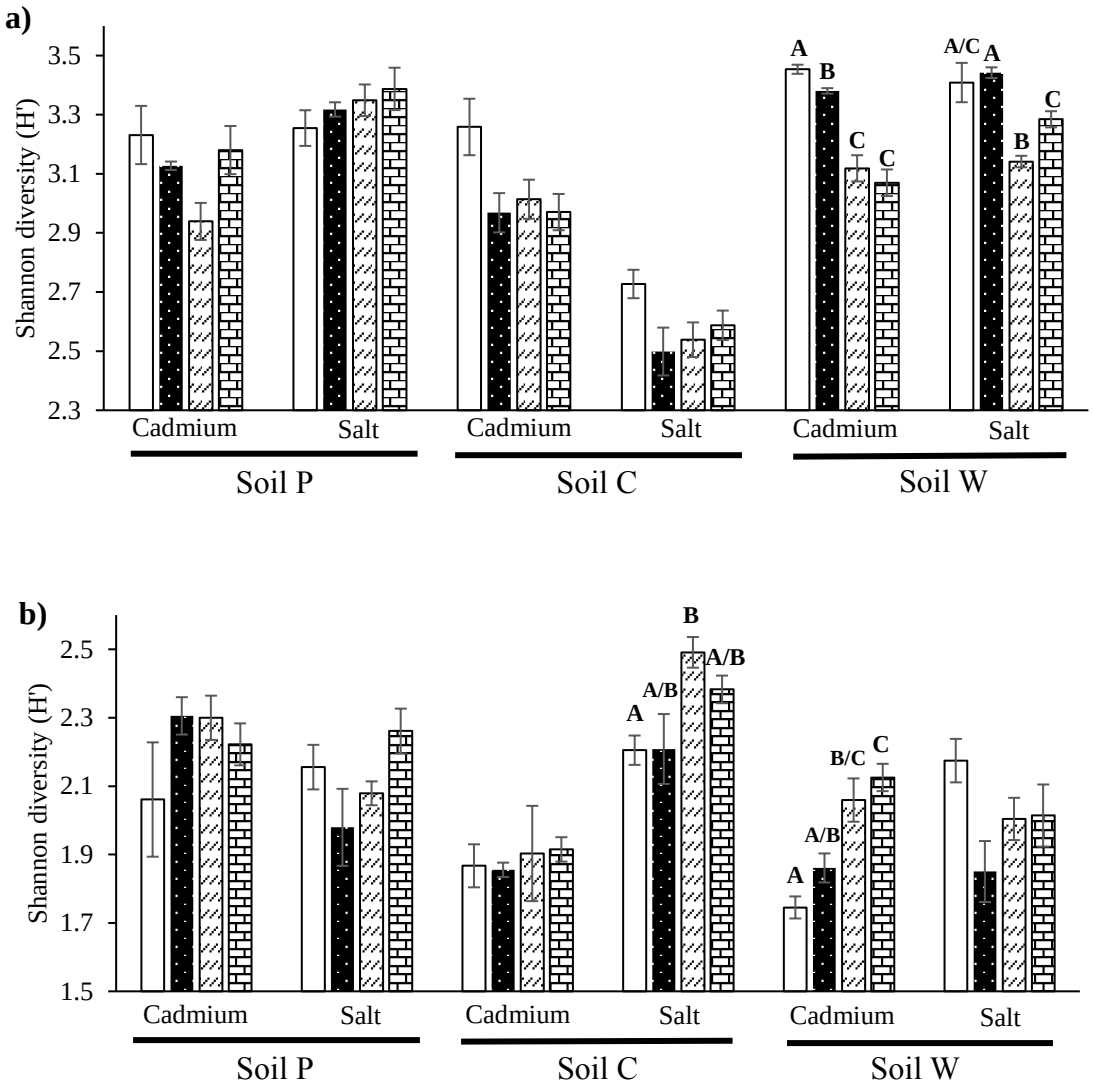
Fungal communities within control samples (dilute ethanol only) and low stressor (cadmium or salt) treatments showed no significant difference in overall species richness and evenness (H') across all soils for both stressors (Supplementary Figure 2.7.2b). In contrast to bacteria, diversity increased in all fungal assemblages impacted by melatonin under low stress conditions. A significant shift in diversity of fungal communities relative to the low stress treatment was only recorded in soil C under low salt stress upon treatment with low melatonin. Communities within soil W under low cadmium stress increased in diversity in response to low melatonin, however only significantly ($p < 0.05$) when compared to the control (Supplementary Figure 2.7.2b).

Similar patterns were observed under high stress treatments, with bacterial communities decreasing in diversity in response to melatonin whereas fungi responding to melatonin by increasing in diversity (Supplementary Figure 2.7.3). Treatments of

melatonin with high cadmium or salt in soil W resulted in significant decreases in bacterial diversity in comparison with the high stressor treatments, whereas only one fungal community (soil W under high cadmium stress) increased significantly ($p < 0.05$) in diversity under the same relative comparison. Otherwise, melatonin showed no effect on microbial community diversity indices under high stress conditions.



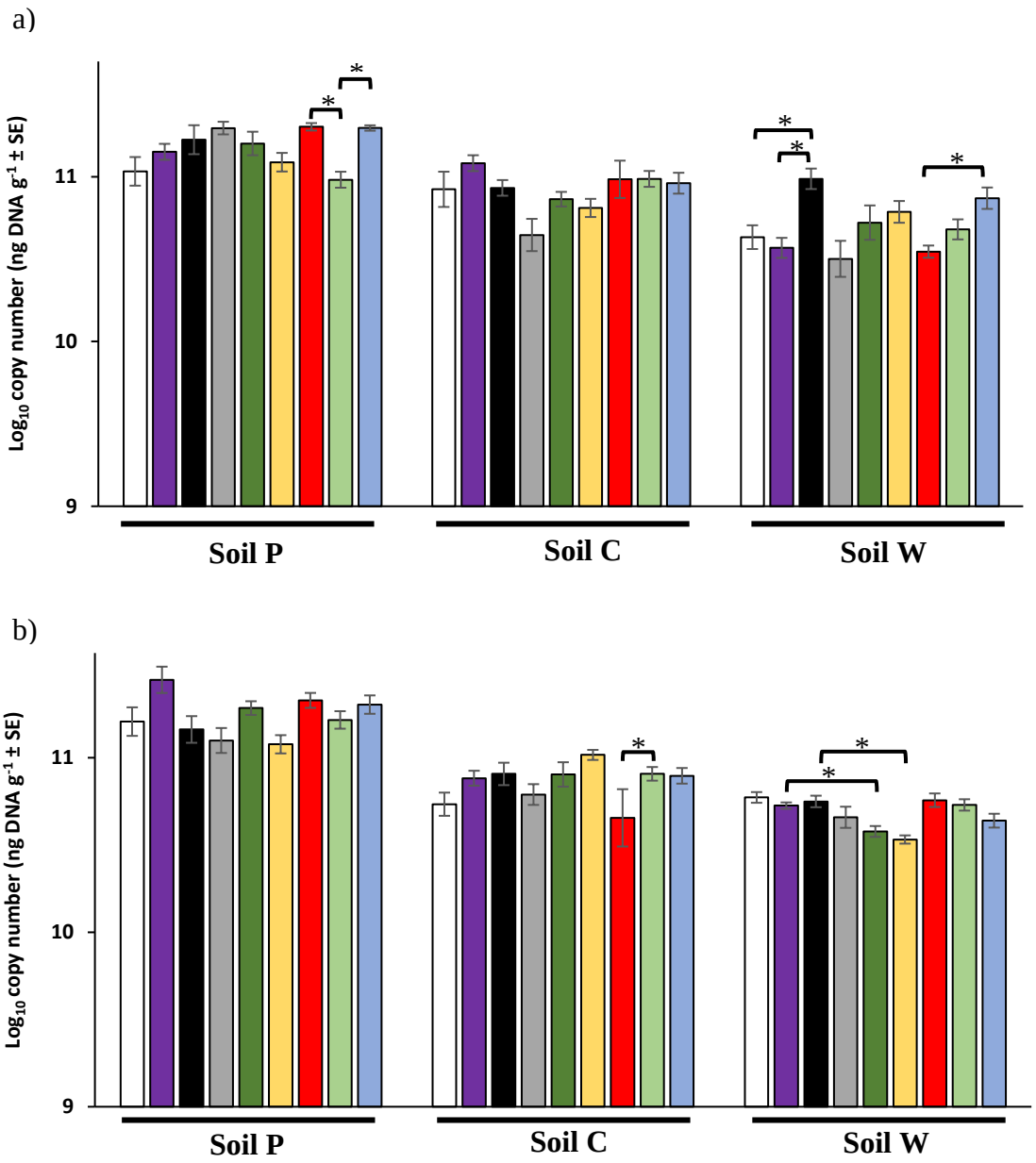
Supplementary Figure 2.7.2: Mean OTU abundance and evenness based upon OTU diversity (Shannon's index – H') for a) bacterial and b) fungal communities showing the effects of melatonin under low stressor (cadmium or salt) conditions. ☐ = Control (no melatonin, no stress); ⬚ = No melatonin + low stress; ▨ = Low melatonin + low stress; ⬚ = high melatonin + low stress. Shannon's index was calculated in the vegan R software using OTU counts and relative abundances. Bars represent standard error (n = 4). Letters denote significant ($p < 0.05$) differences between treatments within an individual soil experiment as determined by Wilcoxon non-parametric analyses. All treatments and controls were composed of a standardised amount of dilute ethanol per soil.



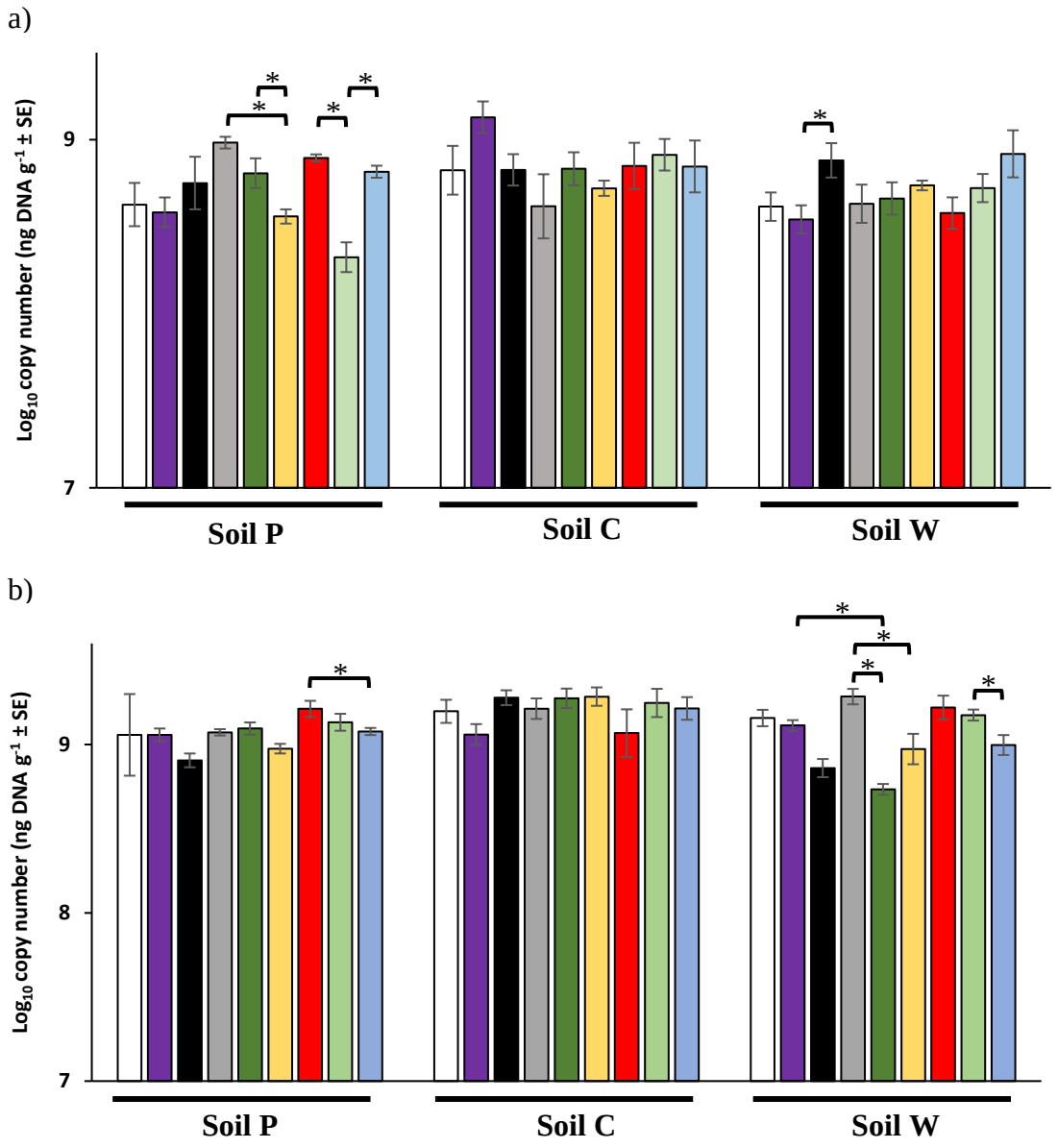
Supplementary Figure 2.7.3: Mean OTU abundance and evenness based upon OTU diversity (Shannon's index – H') for a) bacterial and b) fungal communities showing the effects of melatonin under high stressor (cadmium or salt) conditions. ☐ = Control (no melatonin, no stress); ▪ = No melatonin + high stress; ▨ = Low melatonin + high stress; ▦ = high melatonin + high stress. Shannon's index was calculated in the vegan R software using OTU counts and relative abundances. Bars represent standard error (n = 4). Letters denote significant ($p < 0.05$) differences between treatments within an individual soil experiment as determined by Wilcoxon non-parametric analyses. All treatments and controls were composed of a standardised amount of dilute ethanol per soil.

2.7.5 Effects of treatments on total bacteria and fungi gene copy numbers (qPCR analysis)

Total microbial gene copy numbers were assessed to inform about changes in microbial biomass in response to treatments (Supplementary Figures 2.7.4 & 2.7.5). Soils treated with melatonin show no changes in bacterial biomass compared to control soil samples (i.e. not treated with melatonin or stressors) for soils P and W, whereas high melatonin alone resulted in a significant increase ($p < 0.05$) in soil C, however only at sampling timepoint T0 (Supplementary Figure 2.7.4). Total fungal biomass was unaffected by melatonin across all three soils in the absence of an abiotic stressor (Supplementary Figure 2.7.5). The effects of melatonin on bacteria and fungi under abiotic stress varied according to stressor concentration and soil type. For example, under low cadmium stress conditions, melatonin application resulted in a significant increase ($p < 0.05$) in total bacteria in soil W, while in soil P, melatonin reduced bacterial gene copy numbers (Supplementary Figure 2.7.4a). In contrast, bacteria numbers were unaffected by melatonin at high cadmium concentration. Fungal gene copy numbers also reduced ($p < 0.05$) upon treatment with melatonin under both cadmium concentrations in soil P, however no effects were observed in soils C or W for either stressor condition (Supplementary Figure 2.7.5a). Under low salt stress, total bacterial gene copy numbers increased ($p < 0.05$) to the availability of melatonin in soil C, with no shifts observed in the other soil communities under low or high salt stress (Supplementary Figure 2.7.4b). Total fungal gene copy numbers decreased in soils P and W under salt stress upon the availability of melatonin, however no differences were observed in soil C. (Supplementary Figure 2.7.5b).



Supplementary Figure 2.7.4: Quantitative PCR estimation of bacterial gDNA copy numbers for communities treated with a) cadmium and b) salt stressors. Asterisks (*) represents significant differences ($p < 0.05$) in copy number for communities treated with melatonin-only compared to control samples or high / low stressor (cadmium or salt) in the presence vs absence of melatonin as determined by pairwise Wilcoxon non-parametric analyses. Treatments: ☐ = Control; ■ = low melatonin; ■ = high melatonin; ■ = high stress; ■ = low melatonin + high stress; ■ = high melatonin + high stress; ■ = low stress; ■ = low melatonin + low stress; ■ = high melatonin + low stress. Values (± SE) are reported as mean of n = 4 biological replicates based on an average weight of 0.275g soil per sample. All treatments and controls were exposed to a standard amount of dilute ethanol per soil.



Supplementary Figure 2.7.5: Quantitative PCR estimation of fungal gDNA copy numbers for communities treated with a) cadmium and b) salt stressors. Asterisks (*) represents significant differences ($p < 0.05$) in copy number for communities treated with melatonin-only compared to control samples or high / low stressor (cadmium or salt) in the presence vs absence of melatonin as determined by pairwise Wilcoxon non-parametric analyses. Treatments: ☐ = Control; ■ = low melatonin; ■ = high melatonin; ■ = high stress; ■ = low melatonin + high stress; ■ = high melatonin + high stress; ■ = low stress; ■ = low melatonin + low stress; ■ = high melatonin + low stress. Values (± SE) are reported as mean of n = 4 biological replicates based on an average weight of 0.275g soil per sample. All treatments and controls were exposed to a standard amount of dilute ethanol per soil.

2.7.5.1 Melatonin and/or stressor treatments altered microbial community similarities

Analysis of dissimilarity of microbial community structures between control and treatment (melatonin and/or stressor) soil samples showed varying trends for both bacteria and fungi (Ranjard *et al.*, 2001; Chow *et al.*, 2013) (Supplementary Table 2.7.9). Under low salt conditions, melatonin-treated bacterial samples (green highlight) showed a dramatic decrease in dissimilarity to control compared to low salt only (yellow highlight) samples across all three soils (P: 7.6%; C: 33.5%; W: 19.7%) (Supplementary Table 2.7.9a). However, this trend was not observed at high salt conditions for bacterial samples, as melatonin had little effect on the relative differences in dissimilarity, in some cases further increasing dissimilarity. Under high cadmium treatments, melatonin further increased dissimilarity to control compared to the high cadmium-only samples for bacteria, with variable results observed under low cadmium conditions. For fungal communities, changes in dissimilarity under abiotic stress conditions did not follow this same trend at all, with varying patterns across the three soils.

Supplementary Table 2.7.9: Dissimilarities (%) of a) bacterial and b) fungal communities treated with melatonin and/or stressor in comparison to community structures within control samples as determined by SIMPER using Bray Curtis dissimilarity analysis. Colour codes indicate changes in dissimilarities in bacterial communities under low salt stress conditions between samples treated with high melatonin (green) in comparison to no melatonin (yellow).

a)

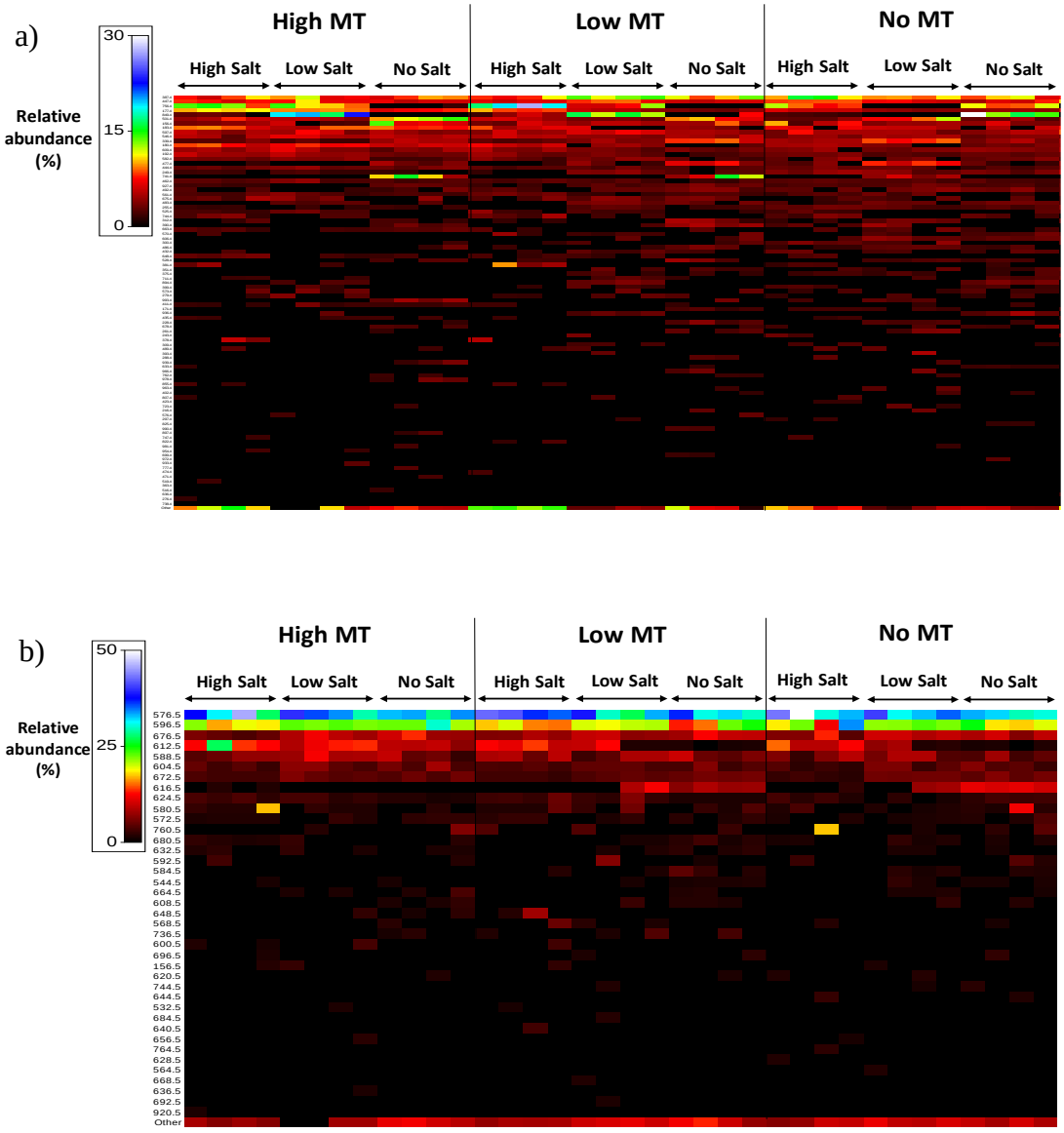
	Soil P			Soil C			Soil W		
	High MT	**Low MT**	**No MT**	**High MT**	**Low MT**	**No MT**	**High MT**	**Low MT**	**No MT**
High Cd	42.53	40.78	37.98	41.16	40.17	40.97	53.83	52.97	41.22
Low Cd	43.45	40.83	44.3	29.15	31.75	34.02	49.35	34.44	34.42
No Cd	41.3	39.89		27.13	31.76		46.58	28.1	
High salt	36.28	32.84	35.87	35.63	36.54	31.81	41.25	41.75	37.02
Low salt	32.54	36.97	40.17	29.45	43.62	62.98	34.14	24.98	44.67
No salt	37.57	40.57		61.71	61.58		50.46	48.05	

b)

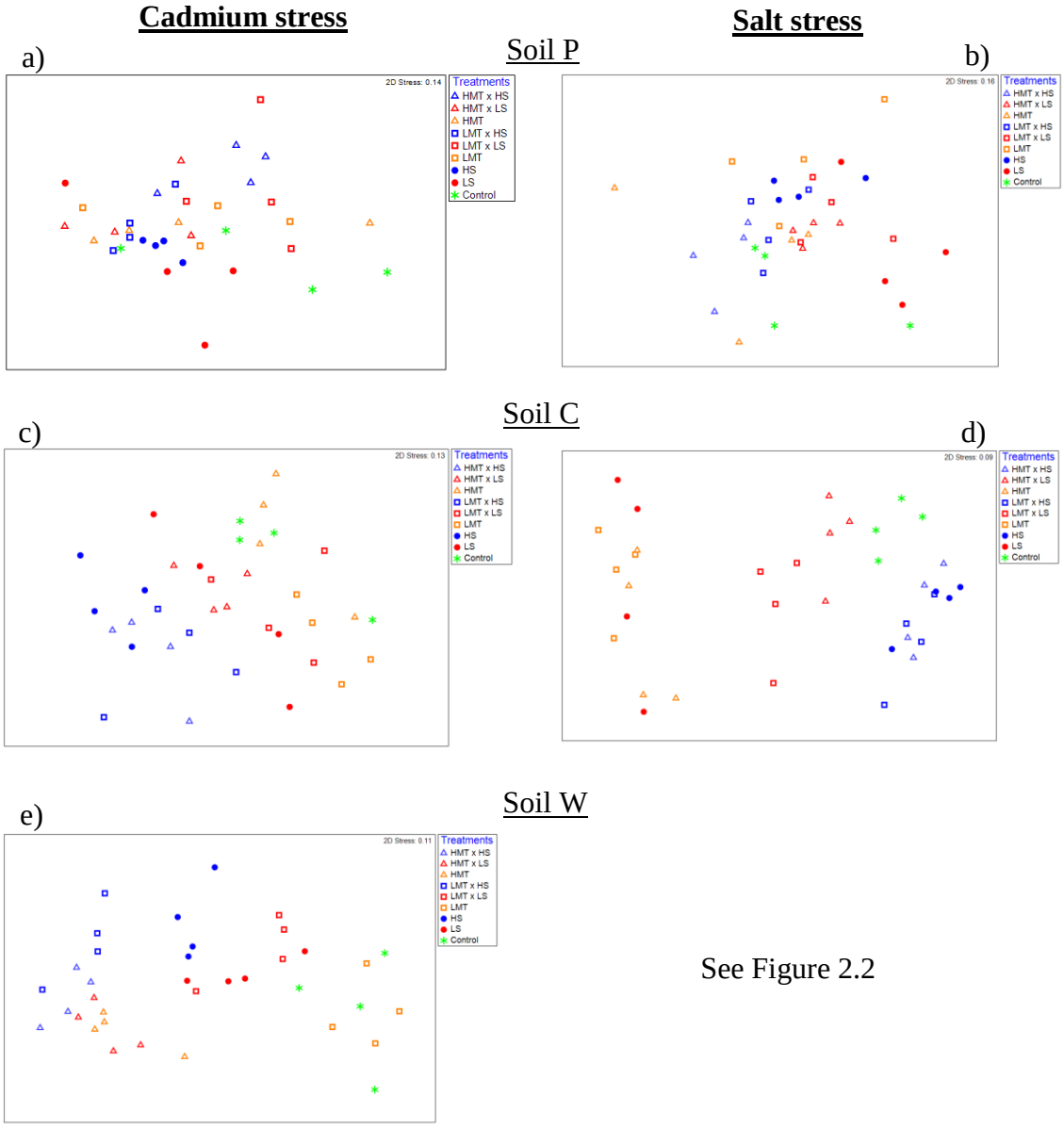
	Soil P			Soil C			Soil W		
	High MT	**Low MT**	**No MT**	**High MT**	**Low MT**	**No MT**	**High MT**	**Low MT**	**No MT**
High Cd	27.94	29.64	26.74	27.16	24.44	18.76	35.59	39.05	27.81
Low Cd	26.87	31.33	31.8	21.74	20.1	19.85	34.8	31.54	30.32
No Cd	26.24	25.16		23.77	28.84		33.72	28.54	
High salt	24.34	22.88	19.55	26.85	25.96	26.21	29.55	29.79	32.16
Low salt	21.01	21.56	21.4	22.2	26.97	26.74	26.66	21.73	20.64
No salt	25.09	21.95		20.29	21.71		26.24	18.2	

MT: melatonin; Cd: cadmium. All treatments and controls were composed of a standardised amount of dilute ethanol per soil. n=4 replicates per treatment.

2.7.6 Responses of microbial communities to treatments

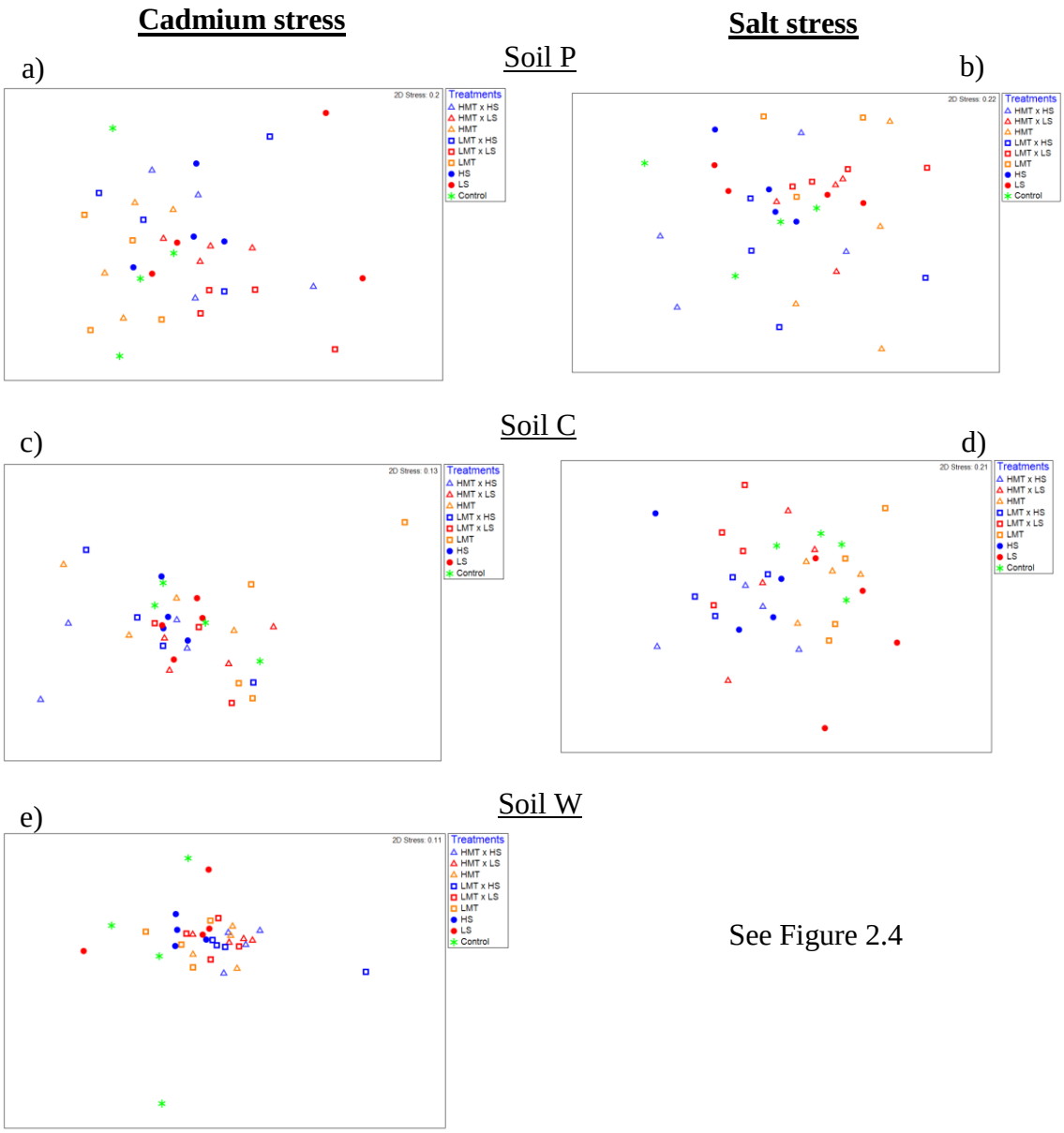


Supplementary Figure 2.7.6: Heatmaps of a) bacterial and b) fungal community compositions for treatments with melatonin and salt in soil W. OTU identifications are provided in rows and columns represents replicate samples (n=4) for various treatments. Relative abundances (%) of individual OTU's are indicated by a colorimetric scale ranging from 0-30% for bacterial and 0-50% for fungal samples. Singletons and OTU's less than 1% relative abundance were removed and classified within the category 'Other', listed as the final row. MT: melatonin. All treatments and controls were composed of a standardised amount of dilute ethanol.



See Figure 2.2

Supplementary Figure 2.7.7: Non-metric multidimensional scaling (nMDS) ordination displaying Bray-Curtis similarities for bacterial samples within soils P (a & b), C (c & d); and W (e) for various treatments of melatonin and stressor based upon community compositions determined by ARISA fingerprinting analysis. Low 2D stress values indicate high quality ordination plots. Relative proximity of replicates reflects high community similarity within the same treatments for bacterial communities. Water replaced salt treatment and dilute ethanol replaced melatonin treatments in respective control samples. All treatments and controls were composed of a standardised amount of dilute ethanol. (HMT: High melatonin; LMT: Low melatonin; HS: High stress; LS: Low stress; HMT x HS: High melatonin with high stress; HMT x LS: High melatonin with low stress; LMT x HS: low melatonin with high stress; LMT x LS: Low melatonin with low stress).



See Figure 2.4

Supplementary Figure 2.7.8: Non-metric multidimensional scaling (nMDS) ordination displaying Bray-Curtis similarities for fungal samples within soils P (a & b), C (c & d); and W (e) for various treatments of melatonin and stressor based upon community compositions determined by ARISA fingerprinting analysis. Low 2D stress values indicate high quality ordination plots. Relative proximity of replicates reflects high community similarity within the same treatments for bacterial communities. Water replaced salt treatment and dilute ethanol replaced melatonin treatments in respective control samples. All treatments and controls were composed of a standardised amount of dilute ethanol. (HMT: High melatonin; LMT: Low melatonin; HS: High stress; LS: Low stress; HMT x HS: High melatonin with high stress; HMT x LS: High melatonin with low stress; LMT x HS: low melatonin with high stress; LMT x LS: Low melatonin with low stress).

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Chapter 3 Melatonin alters bacterial community structures in agricultural soils

Dr. Rohan Lowe (School of Molecular Science, La Trobe University, Melbourne, Australia) contributed 20% to the data analysis component of this study. All experimental work, data interpretation, written presentation and the remaining 80% of data analysis were provided by Andrew Madigan.

3.1 Introduction

Soil microbial communities are a key component of terrestrial ecosystems (Wall and Virginia, 1999; Yao *et al.*, 2000; Wahid *et al.*, 2016). Various bacterial functional groups, including nitrogen-fixing bacteria and plant growth-promoting rhizobacteria (PGPR), contribute to plant health and crop productivity by providing essential minerals and nutrients to plants (Brussaard *et al.*, 2007; Fierer *et al.*, 2007b; Chanda *et al.*, 2016). Plants directly influence microbial community structure and function within the rhizosphere by providing sugars, amino acids and various secondary metabolites, such as indoleamines (e.g. auxins), secreted into the soil as root exudates (Badri and Vivanco, 2009; Dennis *et al.*, 2010). In turn, alteration of microbial community structure can have both direct and indirect effects on plant physiology and development, such as altering the vegetative period duration, plant growth rate or stress tolerance levels (Poupin *et al.*, 2013; Rincon-Florez *et al.*, 2013; Chanda *et al.*, 2016).

Melatonin (N-acetly-5-methoxytryptamine) is an indoleamine produced ubiquitously across all domains of life (Hardeland, 2015; Manchester *et al.*, 2015). Melatonin has been shown to have a diverse range of beneficial physiological effects on plants that may have promising applications in future agriculture practices: enhancing seedling growth and crop yield (Zhao *et al.*, 2015a); altering root architecture and development as well as enhancing root biomass (Chen *et al.*, 2009; Hernández *et al.*, 2015; Zhao *et al.*, 2015a) and seed germination (Zhang *et al.*, 2013); delaying important processes such as leaf senescence (Wang *et al.*, 2013; Zhang *et al.*, 2017) and fruit decay (Gao *et al.*, 2016); promoting fruit ripening (Sun *et al.*, 2016). Melatonin has been described in various microorganisms including bacteria such as proteobacteria and cyanobacteria (Manchester *et al.*, 1995; Hardeland, 1999; Muszyńska *et al.*, 2011; Muszyńska and Sułkowska-Ziaja, 2012; Tan *et al.*, 2014; Vigentini *et al.*, 2015; Jiao *et al.*, 2016; Ma *et al.*, 2017). However, to date, no data has been reported regarding responses of bacterial communities to melatonin in agricultural soils. Melatonin has been shown to reduce the

infection capability of some bacterial pathogens in plants (Zhao *et al.*, 2015b; Chen *et al.*, 2019), and has shown antimicrobial properties in some *in-vitro* studies (Tekbas *et al.*, 2008; Arnao and Hernández-Ruiz, 2015).

The ARISA analyses conducted in chapter 2 demonstrated that exogenous melatonin altered bacterial community structures under abiotic stress (cadmium or salt) conditions. Next generation sequencing (NGS) of bacterial communities was subsequently conducted on the same samples that were treated with melatonin-only from two of the agricultural soils (soil C and W). Due to limited funding and a general lack of responsiveness to treatments compared to bacteria, NGS was not conducted on fungal samples. Concurrently, the bacterial samples associated with soil P showed no significant responses to melatonin only according to ARISA (Supplementary Table 2.7.8) and were thus not included in further analysis by NGS. Preparation of soil samples prior to treatments was an important aspect of this study. Both soils were stored in oxygen-poor conditions for four and five months (Figure 2.1). Soils were subsampled to investigate the responses of differentially aged bacterial communities to melatonin. It was expected that this aging regime would change the initial microbial communities and potentially shift selection pressures amongst the populations (Kelly *et al.*, 1999; Castro *et al.*, 2010; Heijboer *et al.*, 2018; Reese *et al.*, 2018).

Overall, the aim of the research conducted in this chapter is to identify the soil bacteria responsive to exogenous melatonin. It was hypothesised that: 1) melatonin alters bacterial community structures in soil; 2) bacterial communities shift between sampling timepoints for the same soil. This shift will in turn result in different responses by soil bacteria to melatonin at both timepoints; 3) soil microbes respond differently to high and low concentrations of melatonin; 4) melatonin alters the abundance of soil PGPRs within the soil communities.

3.2 Materials & method

3.2.1 Soil sampling and melatonin treatments

All soil samples associated with melatonin-only treatments (i.e. no chemical stressors) from soils C and W that were analysed by ARISA in chapter 2, were further analysed by next generation sequencing. All preparations, treatments and conditions as described in Sections 2.3.1 & 2.3.2.

3.2.2 Microbial community analysis – Illumina MiSeq

Total soil DNA was extracted from 0.25-0.3 g of soil subsamples using PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., California, U.S.A.) according to manufacturer's instructions. MiSeq sequencing was conducted on the V3-V4 of the 16S rRNA gene for bacteria (Yarza *et al.*, 2014; Lazarevic *et al.*, 2016). Sequenced bacterial samples were prepared with barcoded primer sets according to protocols outlined by Illumina for 16S Metagenomic Library sequencing (Dong *et al.*, 2016). Bacterial primers included 341 F (5'-CCT ACG GGN GGC WGC AG-3') and reverse primer 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3'). PCR reactions were performed in a total volume of 25 µl comprising of 2.5 µl gDNA (5 ng / µl), 12.5 µl 2 x KAPA Hifi HotStart ReadyMix (Fisher Scientific, Melbourne, Australia) and 5 µl each of 1 µM forward and reverse primers. Bacterial PCR amplification was conducted according to Illumina protocol (Illumina, San Diego, USA) under a temperature profile of 95°C for 3 min, followed by 25 cycles of amplification at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final extension of 72°C for 5 min. Negative controls indicated any contamination issues. PCR products were cleaned with AMPure XP beads following inspection of quality on 1.5% agarose gels. Dual indices and Illumina sequencing adaptors were attached to amplicons using Nextera XT Index Kit (Illumina, San Diego, USA). Library quantification of cleaned PCR products was conducted on a Qubit 2.0 Fluorometer using a Qubit dsDNA BS Assay Kit (ThermoFisher, Waltham, Massachusetts, USA). Samples were standardised to a concentration of 4 nM and pooled, followed by library submission to MiSeq^{TM} platform (MiSeq Reagent Kit v3; Illumina, San Diego, USA) with 600 cycles of paired-end (2 x 300bp) sequencing.

3.2.3 Bioinformatics & Statistical analysis

Single end reads of the amplified V3-V4 regions for bacteria were merged and subsequently analysed using USEARCH v 10.0.240 (Edgar, 2010). This involved quality filtering, chimeric removal and discarding reads with total expected errors greater than 1.0. Sequences were dereplicated and operational taxonomic units (OTU's) clustered with 97% similarity cut-off. The datasets 16S rRNA RDP training set v16 (Edgar, 2018) was downloaded in July 2018 and used for taxonomic identification of bacterial sequences respectively. Total community richness was assessed by species rarefaction curves and species accumulation plots, generated in the R library *vegan* v2.52 using R v3.5.1 (The R Foundation for Statistical Computing, Boston, USA).

Bacterial data were rarefied to 73,000 sequences per sample and analysed using Bray-Curtis dissimilarity matrix. Permutational multivariate analysis of variance (PERMANOVA) assessed differences between microbial assemblages due to various melatonin treatments within each soil (Anderson and Walsh, 2013). Diversity indices analysis was conducted in R v3.5.1 on rarefied data to investigate shifts in community diversity due to melatonin. The [R] statistical environment and the Bioconductor package 'EdgeR' was used to identify differentially abundant OTU counts between treatments (Robinson *et al.*, 2009). Count data for each OTU was initially filtered using filterByExpr (default settings) to remove OTUs with a low number of counts, OTUs were required to have at least 85 counts/million (CPM) in 4 of the 48 samples. Library size was normalised (calcNormfactors) before the negative-binomial dispersion was estimated (estimateDisp). A generalised linear model with quasi-likelihood pipeline was then calculated (glmQLFit). Differentially abundant OTUs were identified using glmQLFTest on the model, with contrasts that compared single melatonin treatments to the zero melatonin controls. Comparisons were made between bacterial communities related to melatonin treatments (high and low) and soil type (C and W) at both sampling timepoints (T0 and T1). P-values, log2(fold-change), log2(average CPM per OTU), and counts per million values were calculated along with a false discovery rate (FDR) corrected for multiple tests using the Benjamini-Hochberg method. Principle components were calculated in [R] using the princomp function (covariance matrix) acting on log10-transformed raw counts for OTUs.

3.2.4 Quantitative PCR

Total bacterial DNA copy numbers were measured by quantitative PCR (qPCR) from treated soils (sampled separately to the ARISA (chapter 2) and NGS analysis), to assess overall shifts in abundances between control and melatonin treatments. Bacterial communities were assessed using the qPCR primers and thermal cycling conditions as described in Section 2.3.5.

3.3 Results

3.3.1 Overall

This study investigated bacterial community responses to melatonin (High: 4 mg kg^{-1} soil; Low: 0.2 mg kg^{-1} soil) in two agricultural soils (C and W) at two timepoints related to the soil storage conditions (T0 and T1) (Figure 2.1). After quality control, a total of

6,034,089 high quality bacterial sequences were obtained, ranging from 75,219 to 215,188 per sample. This provided a total of 5,829 operational taxonomic units (OTUs) at ≥ 97% similarity level from the 48 soil samples analysed through Illumina MiSeq sequencing analysis. After filtering, 1,596 out of 5,829 bacterial OTUs were retained and analysed. Samples were rarefied to 73,000 reads per sample for PERMANOVA and diversity indices analysis as well as species accumulation curves. Rarefaction curves for total bacteria OTUs did not plateau, however similar levels were reached in both soils (Figure 3.1 a & b), while species accumulation curves reached saturation in both soil C and W (Figure 3.1 c & d). The bacterial sequencing reads belonged to 18 phyla, with *Actinobacteria* (29.3%), *Proteobacteria* (26.1%), *Acidobacteria* (12.0%), unknown (6.2%) and *Firmicutes* (5.2%) the most dominant (relative abundance > 5%). These phyla were common to all 48 libraries (representing the 48 treated soil samples), accounting for 78.8% of the total reads. Bacterial biomass was not affected by melatonin as quantitative PCR (qPCR) results indicated that melatonin did not significantly alter (Wilcoxon pairwise test: $p > 0.05$) bacterial gene copy numbers in comparison to control samples for communities associated with either soil (Figure 3.2). Bacterial communities associated with control samples from the same soil differed significantly ($p < 0.05$) between sampling timepoints T0 and T1 for soil C ($t = 2.9321$; $p = 0.029$) and soil W ($t = 4.04$; $p = 0.0301$).

3.3.2 Bacterial community responses to melatonin from different agricultural soils

The PCA plot showed clear separation between bacterial communities associated with soil C compared to soil W, independent of the soil treatments (Figure 3.3a). Significant differences ($p < 0.05$) were observed between communities associated with control samples and melatonin-treated samples for both soils, however for communities associated with the sampling timepoint T1 only (Figure 3.3a; Table 3.1). In contrast, control and melatonin-treated samples clustered together for each soil at the first sampling timepoint (albeit relatively widely dispersed in the separate ordination space for each soil - Figures 3.3b & c), with no significant difference ($p > 0.05$) observed between communities associated with control samples and melatonin treated samples (Table 3.1).

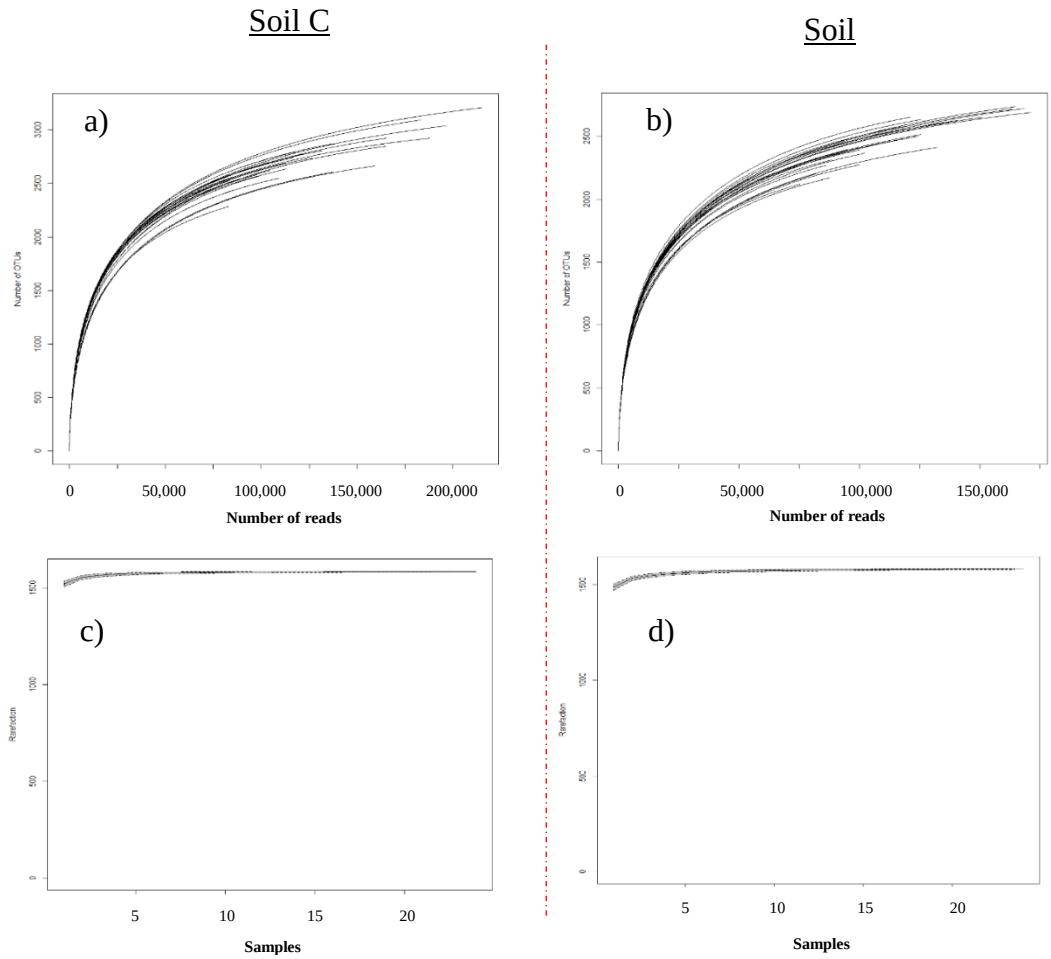

Figure 3.1: Rarefaction curves (a & b) for species richness, and species accumulation (c & d) curves for bacterial communities in soil C (a & c) and soil W (b & d).

Concurrently, exogenous melatonin enhanced ($p < 0.05$) the α diversity (Shannon and Simpson indices) of bacterial communities in soil C and W, however this effect was only observed at the second sampling timepoint (Figure 3.4). Overall, therefore, melatonin modified the abundance of some bacterial communities, altering the α and β diversities in both soils.

3.3.3 High and low concentrations of melatonin differently alter bacterial communities upon the soil sampling time

Changes in the relative abundances of individual bacterial taxa due to low or high melatonin treatments (in comparison to control samples) were assessed using volcano plots. These plots clearly showed that more significant shifts [$\log_{10}$(OTU_FDR) < -2] occurred in both soils at sampling timepoint T1 compared to T0 (Figure 3.5). Interestingly, these plots also showed a relative similarity between overall OTU

responses to high or low melatonin (in comparison with control samples) per soil for communities associated with the timepoint T1. However, analysis by PERMANOVA, taking into consideration the whole set of data, revealed a significant difference ($p < 0.05$) between communities treated with high and low melatonin in soil W at both sampling timepoints; while the same comparisons for soil C communities showed statistically weaker differences ($p = 0.055; 0.084$) (Table 3.1).

Table 3.1: Comparisons of bacterial community compositions treated with high, low and zero (Control) concentrations of melatonin at sampling timepoints T0 and T1 (n = 4 replicates per treatment). Asterisks (*) represent significant differences (P(perm) < 0.05) as determined by PERMANOVA using Bray-Curtis dissimilarity matrices. All treatments were composed of a standardised amount of dilute ethanol per soil (Soil C= 0.052%; Soil W 0.06%). t = t-statistic.

	Soil C				**Soil W**			
	T0		**T1**		**T0**		**T1**	
	t	**P(perm)**	**t**	**P(perm)**	**t**	**P(perm)**	**t**	**P(perm)**
High vs Low	1.786	0.084	1.195	0.055	2.302	**0.028***	1.324	**0.028***
High vs Control	1.048	0.401	3.010	**0.027***	1.932	0.057	4.074	**0.031***
Low vs Control	1.676	0.084	3.035	**0.028***	0.9189	0.858	3.186	**0.028***

3.3.4 Melatonin impacts the abundance of some plant growth promoting rhizobacteria (PGPRs)

Eleven genera of PGPRs, represented by *Bacillus* (averaging 3.03% across all samples), *Burkholderia* (0.745%), *Caulobacter* (0.028%), *Flavobacterium* (0.106%), *Mesorhizobium* (0.045%), *Micrococcinea* (1.116%), *Paenibacillus* (0.467%), *Pseudomonas* (0.0095%), *Rhizobium* (0.005%), *Streptinomyceae* (1.78%) *and Variovorax (0.014%)* (Tables 3.3 and 3.4), were identified in this study using various sources as reference lists for PGPRs (Enebe and Babalola, 2018; Gouda *et al.*, 2018; Majeed *et al.*, 2018; Ramakrishna *et al.*, 2019). *Bacillus* was the most abundant PGPR in all samples at each melatonin or control treatment, independent of soil or sampling timepoint (Tables 3.3 & 3.4). Responses of *Bacillus* to melatonin were highly variable and showed no overall pattern. For example, high melatonin resulted in a decrease (in comparison to the respective control samples) in relative abundance by 6,432 counts per million (CPM; equivalent to 0.643%) in soil C at T0, whereas at T1, high melatonin caused an increase by 451 CPM (0.0451%). In contrast, in soil W, the relative

abundance of *Bacillus* increased by 14,884 CPM (1.488%) for high melatonin treatment at T0, while at T1, the same treatment resulted in a decrease by 6140 CPM (0.614%) under the same respective comparisons (Tables 3.3 & 3.4). *Streptomycineae* was generally the second most abundant PGPR genus across all samples and showed more consistent responses to melatonin compared to *Bacillus*, generally decreasing in relative abundance, however not significantly ($p > 0.05$). Only *Paenibacillus* and *Burkholderia* significantly ($p < 0.05$) changed in response to exogenous melatonin in both soils, with *Paenibacillus* decreasing and *Burkholderia* increasing in relative abundances in comparison to respective control samples (Figures 3.6 & 3.7; Tables 3.3 & 3.4). The relative abundances of *Rhizobium* and *Caulobacter* increased significantly to high melatonin in soil C and W respectively, while *Mesorhizobium* decreased in response to high melatonin in soil C (each in comparison to respective control samples). In all examples, these significant shifts were observed only at sampling timepoint T1.

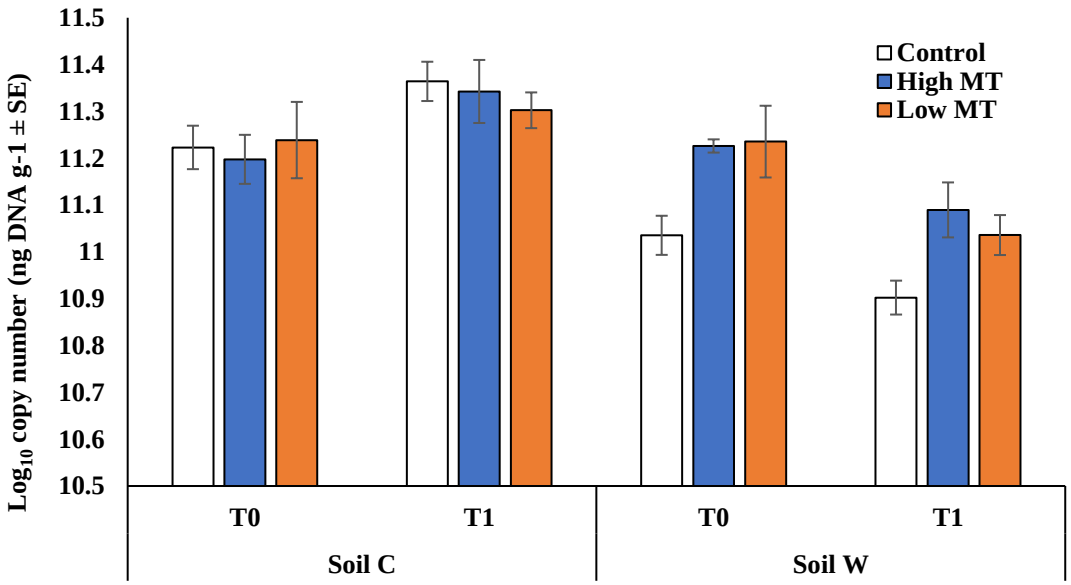

Figure 3.2: Quantitative PCR estimation of bacterial gDNA copy numbers for communities treated with high and low melatonin (MT) at sampling timepoints T0 and T1. Values (± SE) are reported as mean of n = 4 replicates per treatment. All treatments and controls were composed of a standardised amount of dilute ethanol per soil (Soil C= 0.052%; Soil W 0.06%). Pairwise Wilcoxon non-parametric analyses reported no significant differences ($p > 0.05$) in copy number for communities treated with melatonin-only compared to control samples. Soil C represents soil collected 3 weeks post canola harvest. Soil W represents soil collected 3 weeks post fire-blazed wheat harvest.

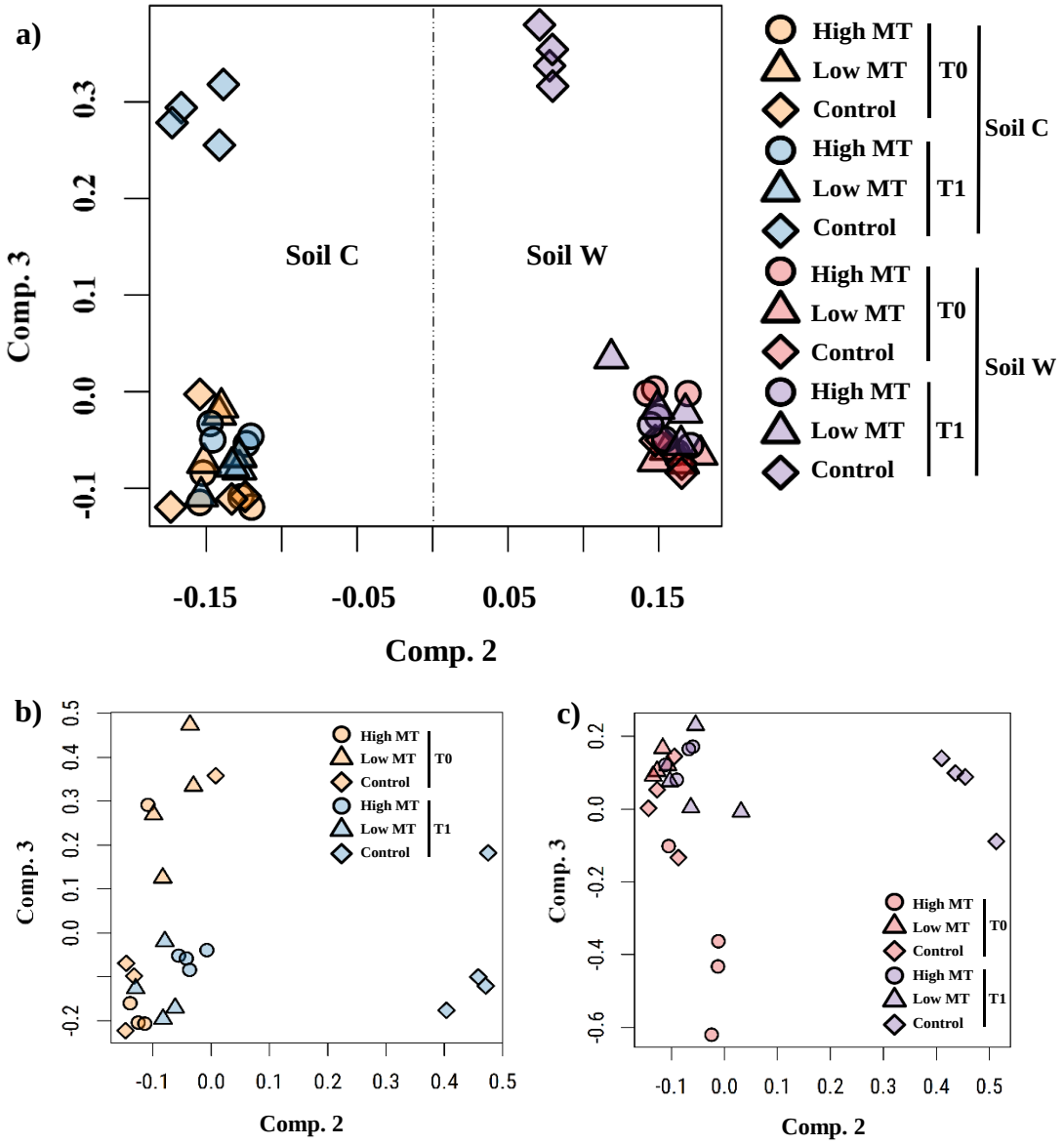

Figure 3.3: Principal components analysis (PCA) of bacterial samples (Principal components [Comp.] 2 and 3) associated with a) soils C and W; b) soil C; and c) soil W. Samples were treated with high, low and zero (Control) concentrations of melatonin (MT) at sampling timepoints T0 and T1 (n = 4 replicates per treatment). All treatments were composed of a standardised amount of dilute ethanol per soil (Soil C= 0.052%; Soil W 0.06%). Soil C represents soil collected 3 weeks post canola harvest. Soil W represents soil collected 3 weeks post fire-blazed wheat harvest.

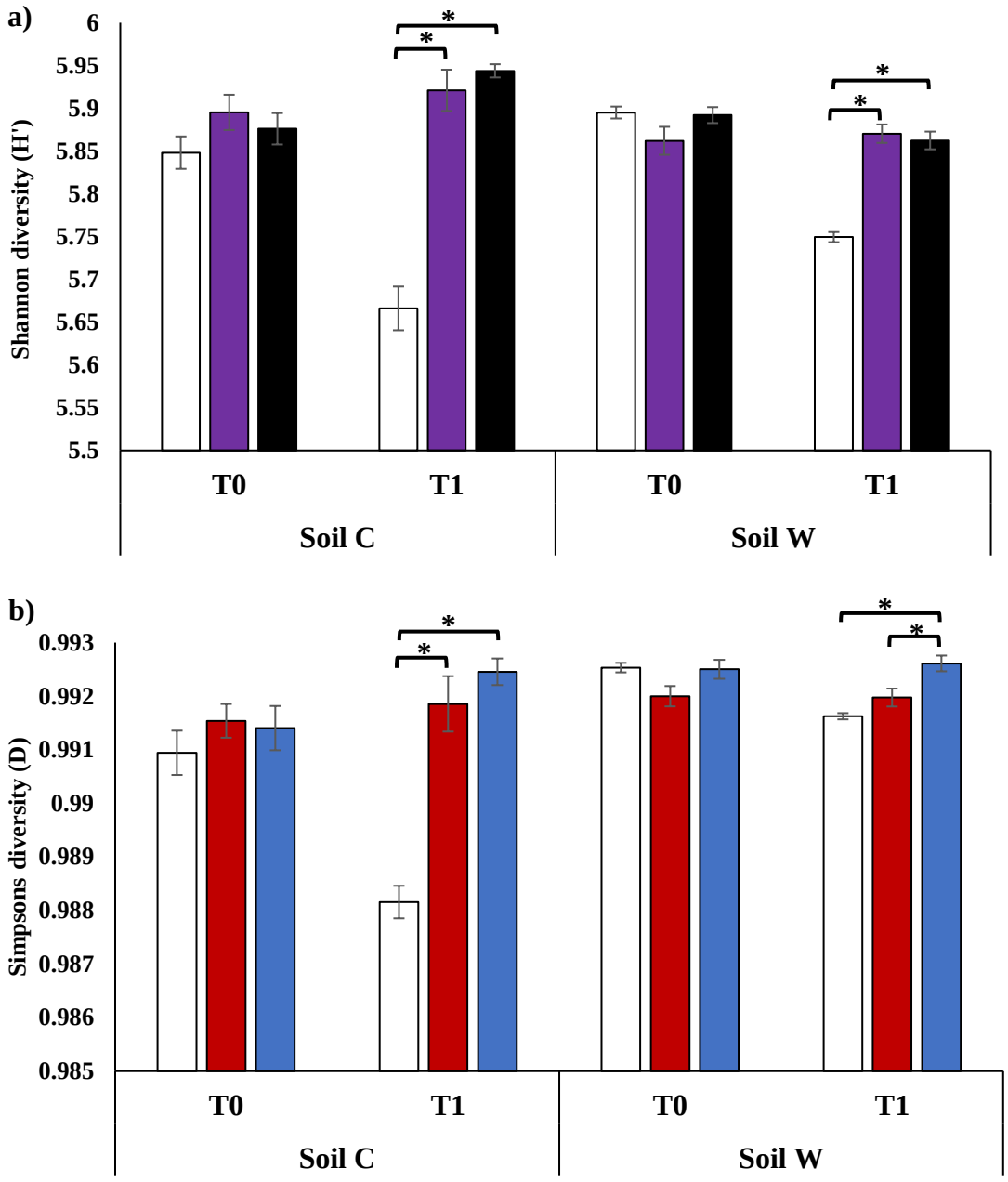

Figure 3.4: Bacterial community responses to melatonin treatments as determined by a) Shannon and b) Simpsons diversity indices for data rarefied to 73,000 reads per sample post filtering. Diversity indices were calculated in the vegan R software using OTU counts and relative abundances. Values (± SE) are reported as mean of n = 4 replicates per treatment. Nonparametric analysis of data by pairwise Wilcoxon rank sum tests in R-determined statistical differences (* representing $p < 0.05$) amongst treatments from the same soil and sampling timepoint. Bars represent standard error (n = 4). All treatments and controls were composed of a standardised amount of dilute ethanol per soil. Control = □; High melatonin = ■ or ■; Low melatonin = ■ or ■

Table 3.2: Changes in relative abundance values (%) of bacterial phyla in response to high or low concentrations of melatonin (in comparison to control [zero] treatments) at sampling timepoints T0 and T1. Positive results represent increases in relative abundance (% within total population) of phyla upon melatonin treatment and vice versa. Significant shifts (False Discovery Rate < 0.05) are highlighted in orange. Soil C represents soil collected 3 weeks post canola harvest. Soil W represents soil collected 3 weeks post fire-blazed wheat harvest.

Bacterial Phyla	Soil C				Soil W				Overall
	T0		T1		T0		T1		
	High vs Zero	Low vs Zero	High vs Zero	Low vs Zero	High vs Zero	Low vs Zero	High vs Zero	Low vs Zero	
Gemmatimonadetes	1.0*	0.1	1.4*	1.8*	-0.7	0.2	2.6*	2.0*	**8.5**
candidate_division_WPS-1	0.1	-1.4	1.4*	2.2*	-1.6	0.3	1.7*	1.7*	**4.4**
Unknown	0.1	-0.2	1.2*	1.8*	-0.1	0.3	0.2*	0.7*	**4.1**
Candidatus_Saccharibacteria	0.5*	-0.1	0.2	0.5	-1.4	<0.1	2.1*	1.8	**3.6**
Acidobacteria	-0.9	-3.9*	2.2	1.4	-2.0	1.0	2.7	2.9	**3.3**
Planctomycetes	-0.5	0.4	0.8*	1.0*	0.3	0.1	0.1	0.4	**2.5**
Verrucomicrobia	0.3	-0.4	0.4	0.5	-0.3	-0.1	<0.1*	0.1*	**0.4**
candidate_division_WPS-2	<0.1	<0.1	<0.1	<0.1	-0.1	0.1	0.1*	0.2	**0.4**
Parcubacteria	<0.1*	<0.1	<0.1*	<0.1	-0.1	0.1	0.1*	0.1*	**0.3**
Armatimonadetes	<0.1	<0.1	<0.1*	<0.1*	<0.1	<0.1	<0.1*	<0.1	**0.2**
Microgenomates	<0.1	<0.1*	<0.1*	<0.1*	<0.1	<0.1	<0.1*	<0.1*	**<0.1**
Chlamydiae	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1*	**<0.1**
BRC1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1*	<0.1	**<0.1**
Cyanobacteria/Chloroplast	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	-0.2*	<0.1	**-0.1**
Chloroflexi	-0.2	-0.1	0.1	<0.1	0.1	-0.1	-0.4*	-0.3*	**-1.0**
Bacteroidetes	<0.1	-1.0	-0.9*	-0.6*	-1.1	-0.1	1.0	0.7	**-2.0**
Actinobacteria	1.4	7.8*	-1.9	-2.7*	8.0*	-1.6	-9.2*	-8.4*	**-6.6**
Firmicutes	-1.2	2.2	-1.2	-2.0*	2.2*	-0.2	-4.7*	-4.1*	**-8.9**
Proteobacteria	-0.6	-3.5	-3.8*	-3.8*	-3.2	0.2	3.5	2.1	**-9.1**

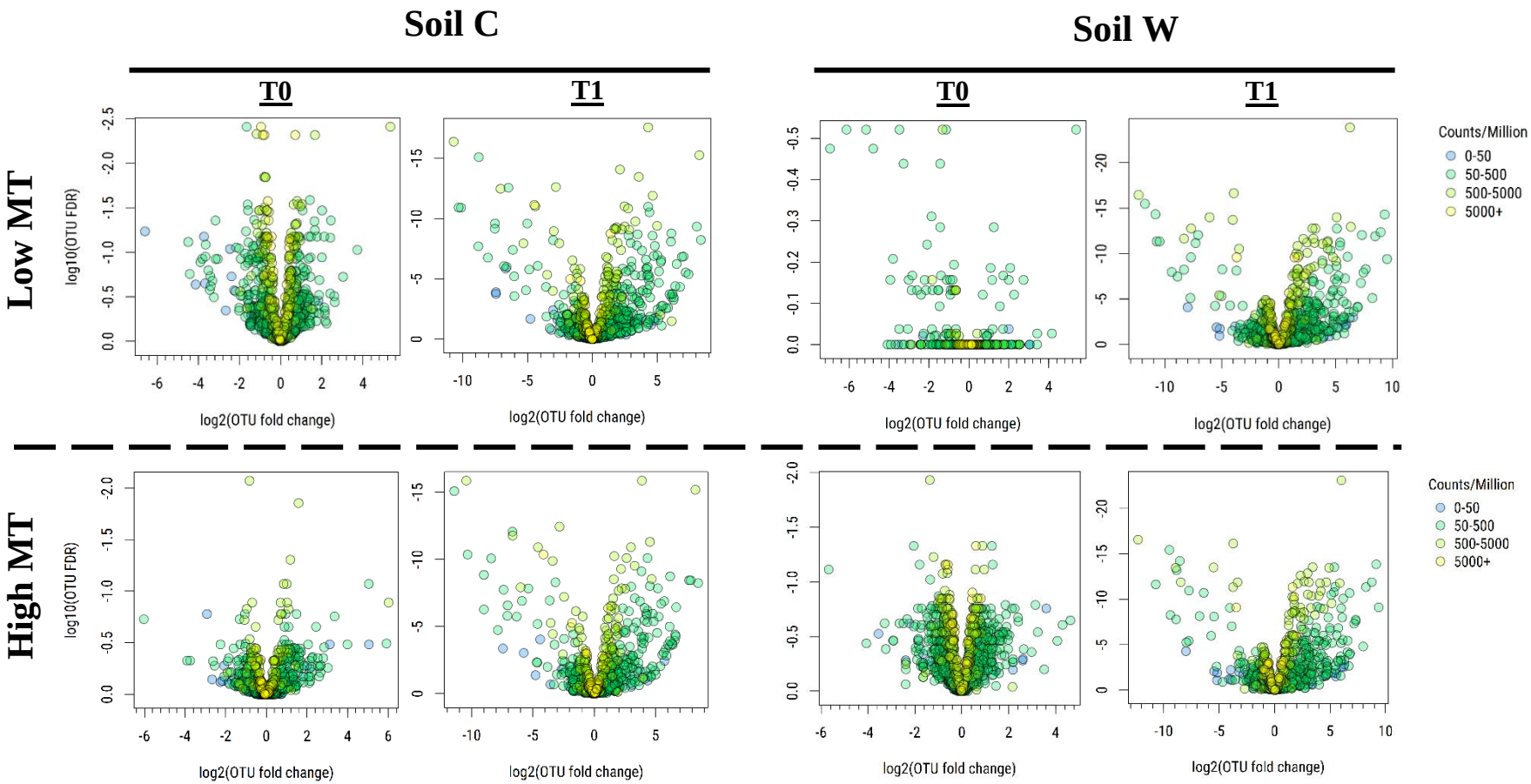

Figure 3.5: Volcano plots of bacterial OTU responses to low or high melatonin treatments (in comparison with control (zero) treatments) within soils C and W at sampling timepoints T0 and T1. A positive fold change represents an increase in OTU relative abundance upon treatment with melatonin and vice versa. MT: Melatonin; FDR: False Discovery Rate.

Table 3.3: Mean counts per million (CPM) of individual plant growth promoting rhizobacteria (PGPRs) in soil C across control (Zero MT), low melatonin (Low MT) and high melatonin (High MT) treatments at sampling timepoints T0 and T1. A positive change (highlighted in blue) represents a mean increase in CPM value upon treatment with melatonin in comparison with the control samples. Negative changes are highlighted in purple. Asterisks (*) represents significant differences (False discovery rate (FDR) < 0.05) between treatments and controls as determined by the Benjamini-Hochberg method.

	T0					**T1**				
	Mean (CPM)			**Change (CPM)**		**Mean (CPM)**			**Change (CPM)**	
Genera	**Zero MT**	**Low MT**	**High MT**	**Low MT**	**High MT**	**Zero MT**	**Low MT**	**High MT**	**Low MT**	**High MT**
Bacillus	37999.7	44483.2	31567.6	6483.5	-6432.2	26779.4	25101.2	27231.0	-1678.1	451.6
Paenibacillus	1069.4	1003.2	830.4	-66.1	-239.0	16553.8	1302.4	1215.9	**-15251.4***	**-15337.9***
Streptomycineae	10358.5	11440.0	15399.6	1081.5	5041.1	12785.5	12065.1	8816.2	-720.4	-3969.3
Micrococcineae	8295.7	10347.7	8960.4	2051.9	664.7	11223.2	9206.0	9471.3	-2017.2	-1751.9
Burkholderia	6469.9	6085.6	5262.1	-384.2	-1207.8	4295.7	5819.3	5366.1	**1523.6***	1070.4
Flavobacterium	1466.7	732.0	1533.6	-734.6	66.9	2850.2	1976.5	1822.3	-873.7	-1027.8
Mesorhizobium	678.7	595.4	944.6	-83.2	265.9	967.8	596.4	406.1	-371.5	**-561.7***
Caulobacter	225.8	179.7	355.6	-46.1	129.8	186.1	286.4	274.6	100.4	88.5
Variovorax	162.6	156.3	216.4	-6.3	53.8	215.3	158.4	196.8	-56.8	-18.5
Pseudomonas	206.9	104.9	475.6	-102.1	268.6	65.0	85.9	91.6	21.0	26.6
Rhizobium	25.7	22.8	274.7	-2.9	248.9	8.3	8.4	133.3	0.1	**125.0***

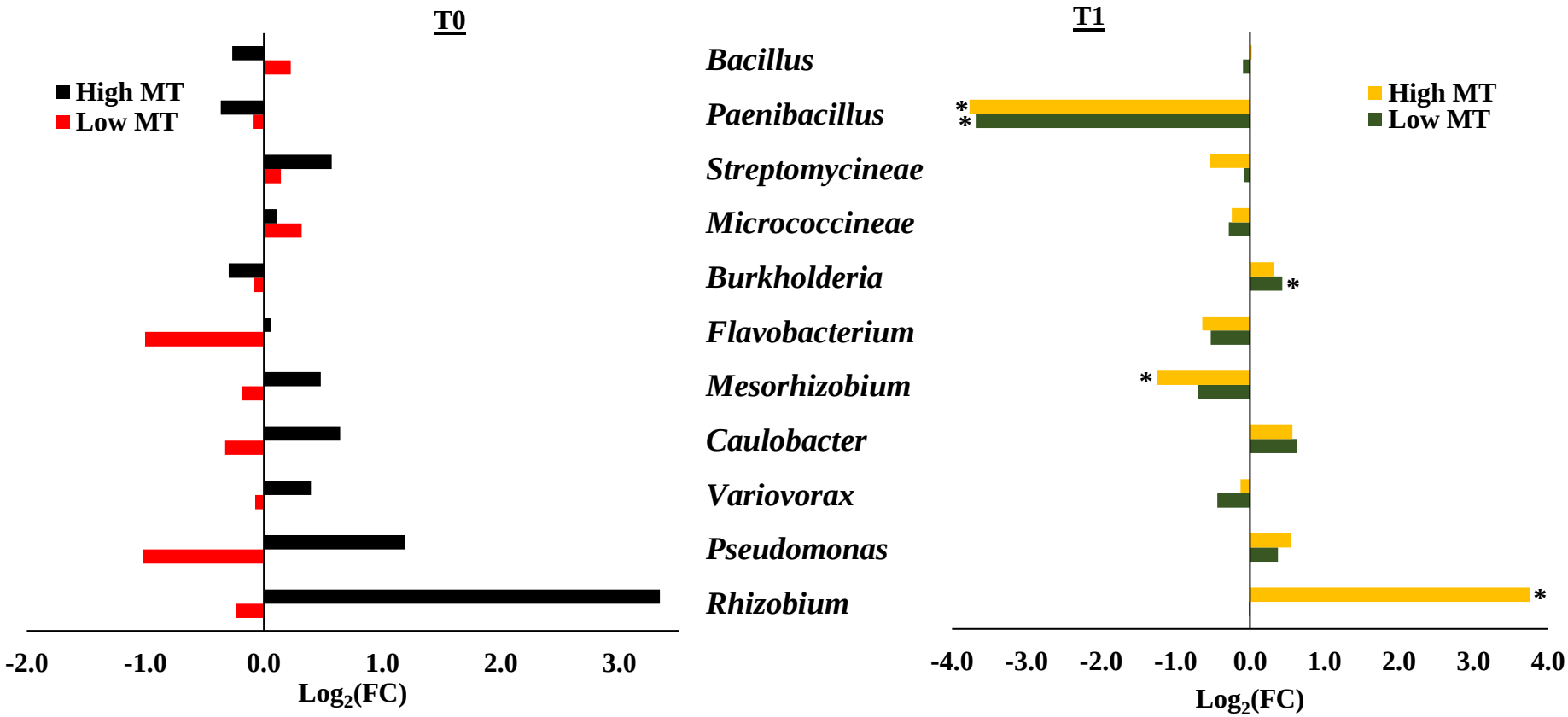

Figure 3.6: Logarithmic fold changes (Log_2FC) of plant growth promoting rhizobacteria (PGPRs) in response to treatments with high and low concentrations of melatonin (MT) in soil C for sampling timepoints T0 and T1. Fold changes were based upon the mean counts per million (CPM) and relative to control treatments with positive changes representing increases in relative abundances upon treatment with melatonin and vice versa. Asterisks (*) represents significant differences (False discovery rate (FDR) < 0.05) between treatments and controls as determined by the Benjamini-Hochberg method.

Table 3.4: Mean counts per million (CPM) of individual plant growth promoting rhizobacteria (PGPRs) in soil W across control (Zero MT), low melatonin (Low MT) and high melatonin (High MT) treatments at sampling timepoints T0 and T1. A positive change (highlighted in blue) represents a mean increase in CPM value upon treatment with melatonin in comparison with the control samples. Negative changes are highlighted in purple. Asterisks (*) represents significant differences (False discovery rate (FDR) < 0.05) between treatments and controls as determined by the Benjamini-Hochberg method.

	T0					**T1**				
	Mean (CPM)			**Change (CPM)**		**Mean (CPM)**			**Change (CPM)**	
Genera	**Zero MT**	**Low MT**	**High MT**	**Low MT**	**High MT**	**Zero MT**	**Low MT**	**High MT**	**Low MT**	**High MT**
Bacillus	23974.2	23783.5	38858.5	-190.7	14884.3	30084.2	29772.5	23944.1	-311.7	-6140.1
Paenibacillus	1006.5	937.1	1207.5	-69.3	201.0	28242.2	1075.4	1586.1	**-27166.8***	**-26656.0***
Streptomycineae	24667.2	22792.7	22195.7	-1874.4	-2471.5	25750.2	25407.6	22384.9	-342.5	-3365.3
Micrococcineae	12998.6	11793.0	16007.1	-1205.6	3008.5	12007.6	11265.8	12373.1	-741.8	365.5
Burkholderia	10213.3	10179.3	8171.3	-33.9	-2041.9	4799.7	13683.1	9005.1	**8883.4***	**4205.4***
Flavobacterium	330.2	310.8	389.5	-19.4	59.3	554.1	423.8	317.4	-130.3	-236.7
Mesorhizobium	270.1	199.9	180.6	-70.3	-89.5	169.6	220.9	214.1	51.3	44.5
Caulobacter	348.0	484.1	270.7	136.1	-77.3	121.6	258.2	372.5	136.5	**250.8***
Variovorax	92.1	98.2	119.1	6.1	27.0	119.7	94.2	77.2	-25.5	-42.5
Pseudomonas	18.6	13.2	26.3	-5.4	7.7	19.1	3.6	32.6	-15.4	13.5
Rhizobium	2.2	8.8	8.3	6.6	6.1	16.9	87.7	2.2	70.8	-14.8

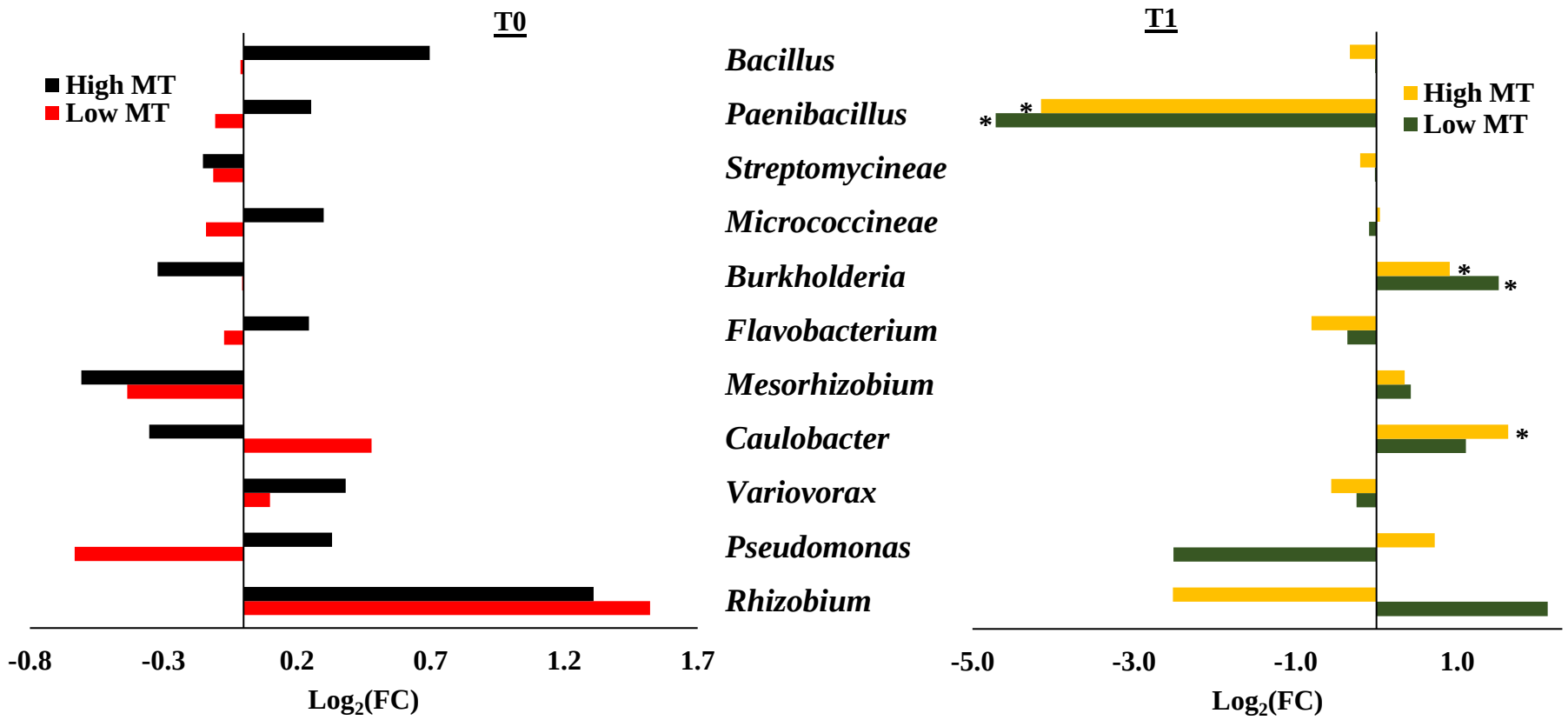

Figure 3.7: Logarithmic fold changes (Log_2FC) of plant growth promoting rhizobacteria (PGPRs) in response to treatments with high and low concentrations of melatonin (MT) in soil W for sampling timepoints T0 and T1. Fold changes were based upon the mean counts per million (CPM) and relative to control treatments with positive changes representing increases in relative abundances upon treatment with melatonin and vice versa. Asterisks (*) represents significant differences (False discovery rate (FDR) < 0.05) between treatments and controls as determined by the Benjamini-Hochberg method.

3.4 Discussion

3.4.1 Overview of investigation

Soil microbial communities are a key component of a healthy ecosystem (Kirk *et al.*, 2004; Wahid *et al.*, 2016), providing essential nutrients and growth requirements directly and indirectly to plant roots (Wall and Virginia, 1999; Yao *et al.*, 2000; Kirk *et al.*, 2004; Wahid *et al.*, 2016). The responses of bacterial assemblages from two agricultural soils to melatonin were assessed at two separate timepoints (T0 and T1) using next generation sequencing (NGS) via Illumina MiSeq Metagenomic Library techniques. Bacterial primers amplified the V3-V4 region of 16S rRNA genes. All treatments contained an equal amount of dilute ethanol per soil (Soil C: 0.052% v/v; Soil W: 0.06% v/v) to ensure that bacterial responses to the dilute solvent were standardised across all samples. Overall, the results showed that exogenous melatonin significantly ($p < 0.05$) altered bacterial community structures in both soils (Table 3.1). Distinctly different bacterial community responses to melatonin were observed at sampling timepoint T0 compared T1 for both soils (Table 3.2; Figure 3.5). This could be interpreted as potentially indicating that soil bacterial community structures and/or some important soil physiochemical characteristics were altered between timepoints T0 and T1 (Kelly *et al.*, 1999; Pesaro *et al.*, 2003; Castro *et al.*, 2010; Heijboer *et al.*, 2018; Reese *et al.*, 2018).

3.4.2 Responses of bacterial phyla to melatonin

Actinobacteria (29.3%), *Proteobacteria* (26.1%) and *Acidobacteria* (12.0%) were the predominant phyla across all samples. This finding is consistent with other soil studies across the world, where these phyla have dominated soils associated with agriculture, grasslands, forests and shrubs (Yadav *et al.*, 2015; Delgado-Baquerizo *et al.*, 2018; Wang *et al.*, 2018; Yu *et al.*, 2019). *Actinobacteria* are drought tolerant bacteria having important ecological roles such as plant decomposition, breaking down plant biomass with cellulolytic enzymes, as well as acting as eukaryotic symbionts (Currie *et al.*, 1999; De Boer *et al.*, 2005; Wang *et al.*, 2012; Lewin *et al.*, 2016). *Acidobacteria* are a diverse and resilient bacterial phylum associated with nutrient cycling across various soil types, including challenging environments such as carbon-poor, low resource or heavily disturbed soils (Fierer *et al.*, 2007a; Huang *et al.*, 2015; Kielak *et al.*, 2016). *Acidobacteria* and *Actinobacteria* are highly sensitive to soil pH, with acidic and neutral soils preferred by either respectively (Lauber *et al.*, 2009). *Actinobacteria* and

Acidobacteria levels have also been shown to increase in rhizosphere soil compared to surrounding topsoil, suggesting they are involved in plant-microbe interactions (Peiffer *et al.*, 2013; Aguirre-Von-Wobeser *et al.*, 2018). *Proteobacteria* are a highly diverse phylum, associated with various roles in soil including nitrogen fixation and plant pathogenesis (Chen *et al.*, 2003; Preston *et al.*, 2005; Li *et al.*, 2019).

In the current study, soil C and W were acidic (pH 4.9 and 4.7 respectively) prior to application of treatments, suggesting an initial soil environment more suited to *Acidobacteria*. However, changes in pH during the treatments were not recorded. Similar overall responses to melatonin by both *Actinobacteria* and *Acidobacteria* were observed. In general, the relative abundances of both phyla increased strongly upon melatonin treatment for both soils at the first sampling timepoint (T0), while for the second sampling point (T1), these phyla were negatively affected by melatonin, showing significant decreases in relative abundances across all melatonin treatments and soils (Table 3.2). In contrast, *Proteobacteria* in soil C responded similarly (negatively) to melatonin at both sampling timepoints, whereas they responded differently in soil W according to the sampling timepoint.

Overall these differences in responses to melatonin at the two sampling timepoints suggest a shift in bacterial community structures over time. PERMANOVA analyses support this inference, showing significant ($p < 0.05$) differences between the control communities associated with timepoint T0 compared to the timepoint T1, for soil C ($t = 2.9321$; $p = 0.029$) and soil W ($t = 4.04$; $p = 0.0301$). Various factors could have been influential in generating a shift in bacterial community structures between these timepoints. For example, the additional four weeks of storage between the sampling timepoints may have affected some soil physiochemical characteristics, which in turn could have reshaped community structures (Kelly *et al.*, 1999; Pesaro *et al.*, 2003; Castro *et al.*, 2010; Heijboer *et al.*, 2018; Reese *et al.*, 2018). Similarly, microbes better adapted to the storage conditions (dehydrated; from aerated to anaerobic; room temperature ($21^{o}C$)) between timepoints may have established a greater dominance in the soils and also influenced the establishment of other bacteria (Evans and Wallenstein, 2012; Huang and Hall, 2017; Zhao *et al.*, 2017; Lu *et al.*, 2018). By altering the communities via controlling soil storage conditions, I was able to explore how changes in communities from the same soil altered the subsequent responses to exogenous melatonin. For both soil communities, these shifts resulted in distinctly different

bacterial community responses to melatonin (Table 3.2; Figure 3.5).The shifts in community structures prior to treatments coupled with the individual and collective effects of melatonin on soil bacteria as well as the complex responses of bacteria to the moisture pulse from a treatment may provide an explanation for different community responses to melatonin in the same soils at different timepoints (Collins *et al.*, 2008). In this study, soil physiochemical characteristics were only analysed prior to treatments at timepoint T0, on dry, untreated soils. No data are available to determine shifts in soil chemistry occurring throughout the experiments.

Only one study to date has reported the effects of exogenous melatonin on soil bacterial community structures (Li *et al.*, 2018). This study investigated a potential role of melatonin in the alleviation of replant disease (of unknown cause) in apple (*Malus* X *domestica*). Higher bacterial diversity indices were observed for melatonin-treated replant soil, with increases in some phyla (1.5 fold or less) such as *Chloroflexi*, *Firmicutes* and *Gemmatimonadetes* and slight decreases (< 1% relative abundances) in *Bacteriodes* and *Verrucomicrobia* (Li *et al.*, 2018). These trends were also observed in the study reported here, in that melatonin increased bacterial diversity in both agricultural soils (sampling timepoint T1 only). In contrast to the community changes in the study reported by Li *et al*. (2018), *Chloroflexi* generally decreased in relative abundances upon melatonin treatments in the current study, and responses of *Firmicutes* were variable. The diversity of soil microbial communities is an indicator of soil quality (Yang and Zhang, 2015). Therefore, it may be inferred that melatonin has a potential to improve the overall quality of a soil, based on the similar findings between both studies. Further studies are required to determine the physiochemical conditions most suitable for melatonin to enhance soil bacterial diversity.

Among the phyla responding to melatonin in the current study, *Gemmatimonadetes* showed the most consistent responses, increasing in relative abundances in both soils at each sampling point (Table 3.2). *Gemmatimonadetes* are resilient bacteria generally adapted to low moisture soils and low nutrient (oligotrophic) environments, with a high tolerance to drought and desiccation (DeBruyn *et al.*, 2011; Park *et al.*, 2017). The consistent positive responses of *Gemmatimonadetes* observed between both studies suggests that melatonin may provide a competitive advantage to this phylum. For example, the resilience of *Gemmatimonadetes* may confer a competitive advantage when conditions become more favorable (e.g. soil hydration), and thus it is possible

that *Gemmatimonadetes* may have utilized melatonin more efficiently compared to other bacteria. Interestingly, *Gemmatimonadetes* utilise the highly destructive ozone-depleting nitrous oxide (N_2O) as part of a non-denitrifying respiratory reaction under anoxic conditions (Zhang *et al.*, 2003; Portmann *et al.*, 2012; Domeignoz-Horta *et al.*, 2016; Park *et al.*, 2017). In this context, melatonin may therefore have been indirectly assisting the reduction of soil N_2O emissions. In the current study, all taxa from *Gemmatimonadetes* were associated with the genus *Gemmatimonas*. Very little is known about this genus as the only species that has been sufficiently characterized to date is *G. aurantiaca* strain T-27. This species is a gram negative, polyphosphate-accumulating obligate aerobe, with the capacity to reduce N_2O (Park *et al.*, 2017). Investigating, how *Gemmatimonadetes* utilize melatonin could provide valuable information regarding the functional roles of melatonin in bacteria and how melatonin is utilized to enhance establishment in a microbial community.

3.4.3 Effect of exogenous melatonin on soil PGPRs

Plant growth-promoting rhizobacteria (PGPRs) provide minerals and nutrients to plants that are essential for growth and development (Brussaard *et al.*, 2007; Fierer *et al.*, 2007b). In the current study, 11 genera of PGPRs were detected (Tables 3.3 & 3.4). Responses of *Bacillus* across both soils, sampling timepoints and melatonin treatments were highly variable, with no clear pattern. In contrast, overall *Pseudomonas* increased in relative abundances in response to high concentration of melatonin whereas, low melatonin concentration had an opposite effect. In general, *Rhizobium* increased in response to melatonin, however it is also important to note the low relative abundance (CPM values: Tables 3.3 & 3.4) of *Rhizobium* in samples, as only a slight change could be recorded as a fold-shift. *Bacillus*, *Pseudomonas* and *Rhizobium species* are particularly important PGPRs due to key functional roles associated with nitrogen fixing and highly efficient phosphate solubilisation (Liu *et al.*, 2015; Igiehon and Babalola, 2018). In addition, all three genera actively produce antibiotics, numerous phytohormones and various secondary metabolites including the indoleamine, IAA, within the rhizosphere (Dazzo *et al.*, 2000; Patten and Glick, 2002; Ghosh *et al.*, 2008; Verma *et al.*, 2010). Interestingly, IAA is structurally very similar to melatonin and acts as a signalling molecule between various bacteria thus altering bacterial interactions and soil community dynamics (Spaepen *et al.*, 2007; Arnao and Hernandez-Ruiz, 2014). *Bacillus* also have been shown to inhibit the growth of plant pathogens as well

as stimulate plant systemic resistance to a biotic stressor (Fira *et al.*, 2018; Miao *et al.*, 2018). Importantly, some *Pseudomonas*, such as *P. syringae*, are pathogenic towards plants (Lee *et al.*, 2014; Shi *et al.*, 2015). However, the overall increase in relative abundance of these genera of PGPRs in response to melatonin treatment suggests that soil-applied melatonin might have an indirect positive effect on plant growth and development, via the enhanced establishment of these important members of the soil environment.

The other PGPRs found in the current study, including *Burkholderia, Caulobacter, Flavobacterium, Mesorhizobium, Micrococcinea, Paenibacillus, Streptomycineae* and *Variovorax* have been reported to have various functional roles in soil, including nitrogen fixing, suppression of plant parasitic nematodes or enhancing plant-microbe interactions via the production of secondary metabolites including various phytohormones (Bhattacharyya and Jha, 2012; Jeyanthi and Kanimozhi, 2018; Mhatre *et al.*, 2019). In this study, all of the above genera of PGPRs were represented in control and melatonin-treated samples for both soils. Of these PGPRs, *Paenibacillus* and *Burkholderia* showed significant ($p < 0.05$) responses to melatonin in both soils. For both genera, these significant shifts were observed only in the soils sampled at T1, with *Paenibacillus* decreasing significantly and *Burkholderia* significantly increasing due to melatonin (in comparison to controls) (Figures 3.6 & 3.7; Tables 3.3 & 3.4).

Burkholderia have been shown to improve nitrogen fixation, assist plant growth, improve plant root and shoot development as well as enhance biotic and abiotic stress tolerance in numerous plants including grape (*Vitis vinifera*), potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) (Nowak *et al.*, 1998; Sharma and Nowak, 1998; Sessitsch *et al.*, 2005; Ait Barka *et al.*, 2006; Tallapragada *et al.*, 2016; Araújo *et al.*, 2017). The significantly ($p < 0.05$) positive responses of this genus to melatonin in soils at timepoint T1 could have potentially beneficial indirect effects on plant growth and development. It is possible that bacteria that may have directly competed with *Burkholderia* in the T0 samples were not as dominant in the T1 samples, therefore resulting in greater abundances in the latter soils.

Paenibacillus in soil have many beneficial roles for plants including nitrogen fixation (Xie *et al.*, 2014), phosphate solubilisation (Das *et al.*, 2010; Marra *et al.*, 2012), iron uptake (Zhou *et al.*, 2016) and phytohormone production (e.g. IAA) (Patten *et al.*,

2013). Importantly, the significant decreases in relative abundance of *Paenibacillus* upon treatment with melatonin is strongly associated to the very high abundance of this genus in control samples. For example, relative abundance levels of *Paenibacillus* in control samples for soil C increased by over 10-fold between sampling timepoints T0 and T1 soil samples, while these levels increased by over 25-fold in soil W under the same comparison (Tables 3.3 & 3.4). Interestingly, the relative abundance levels of *Paenibacillus* in melatonin-treated soils were relatively similar in T0 and T1 samples within the same soil. As discussed above, the overall community shifted significantly ($p < 0.05$) between T0 and T1. It is possible that bacteria that may have directly competed with *Paenibacillus* in the T0 samples were not as dominant in the T1 samples, therefore resulting in greater abundances in the latter soils. In turn, melatonin may have restored the overall community dynamic similar to the T0 samples. Alternatively, it is possible that not all PGPRs are sensitive to melatonin as previously shown for some bacteria (Tekbas *et al.*, 2008; Paulose and Cassone, 2016).

3.4.4 Effects of different melatonin concentrations on bacterial assemblages

The results in the study reported here showed variable responses of bacterial communities to different concentrations of melatonin between both soils (Table 3.2). Collectively, however, this data provides an initial indication that there may be a concentration-dependant shift in soil bacterial communities upon treatment with melatonin (Table 3.1). Interestingly, many plant studies have reported a concentration-dependent effect of melatonin on root architecture and development. For example, low melatonin concentrations (< 1 µM) resulted in enhanced primary root growth or development in *Brassica juncea*, *Triticum aestivum* and *Arabidopsis*, whereas at higher concentrations (e.g. 100 µM), melatonin had a negative effect on root systems (Hernández-Ruiz *et al.*, 2005; Chen *et al.*, 2009; Hernández *et al.*, 2015; Wang *et al.*, 2016). Data from the current study therefore leads to the inference that the concentration-dependent responses by soil bacterial communities to melatonin may potentially be an influencing factor in the concentration-dependent effects of melatonin on root growth and development. These results have implications for future plant-melatonin research studies, where melatonin is supplied to plants via the soil. On the basis of this finding, it is recommended that these future studies should include soil microbiome responses to melatonin as part of the analyses. Notably in the current study, bacterial communities responded differently to melatonin treatments between both

sampling timepoints, suggesting that predicting the effect of melatonin on soil bacteria is a highly complex process. Therefore, in-depth studies across a wide range of soil types and environmental conditions are required to assess if this indoleamine has a viable role in future agricultural practices.

3.4.5 Role of fire or crops in shaping bacterial communities

Analyses in the current study clearly demonstrated separation of bacterial communities on the basis of soil type, indicating distinct communities associated with each soil (Figure 3.3). Interestingly, soils C and W were collected on the same day at sites less than 10 km apart and showed similar soil chemistry profiles to each other. However, soil C was sampled 3 weeks post canola crop harvest, while soil W was sampled 3 weeks post fire blazing of a harvested wheat crop. This therefore suggests that the crops associated with the soils and/or fire blazing activity may have shaped distinctly different soil bacteria communities and their resulting responses to melatonin treatments. The plant species associated with a soil has been shown to be a key factor influencing the bacterial community structure within the rhizosphere (Burns *et al.*, 2015). In particular, root exudate composition strongly shapes microbial assemblages, and can vary considerably according to plant species (Uren, 2000). Fire-altered soil communities exposed to further chemical treatment (e.g. nitrogen fertilisation) have also been shown to develop highly distinct communities, adapted to utilising the changed environmental conditions (Allison *et al.*, 2010; Barreiro *et al.*, 2016). Further research is required to determine the extent to which the crops (canola and wheat) and management practice (fire blazing) may have shaped the associated bacterial communities and the resulting responses to melatonin. This may provide valuable information regarding the soil conditions or crops most suitable for future use of melatonin in agriculture.

3.5 Conclusion

This study demonstrated that soil bacterial communities were significantly ($p < 0.05$) altered by exposure to exogenous melatonin. *Gemmatimonadetes* showed the most positive responses to melatonin, increasing in relative abundances for both soils at each sampling point, while some PGPRs also significantly ($p < 0.05$) responded to exogenous melatonin. *Actinobacteria* and *Acidobacteria* showed similar responses to melatonin at both sampling timepoints, suggesting that melatonin may have a similar

role for both phyla. Community shifts to melatonin treatments differed according to the sampling timepoint for both soils, suggesting that the effects of melatonin on bacterial structures are complex. Further research is required to determine the processes by which soil microbes utilise melatonin, such as enhancing microbial activity or altering competitive advantages, and the implications this may have for potential future applications of melatonin in agriculture. To my knowledge, this is the first report investigating the responses of bacterial communities to melatonin in agricultural soils.

3.6 References

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Chapter 4 Melatonin improves *in vitro* growth of plant pathogenic fungi under ethanol and cold stresses

Mr. Chris Harris (Dept. Animal, Plant and Soil Science, La Trobe University, Melbourne, Australia) conducted 20% of the experimental work (media preparation, marking plates and recording fungal growth). The remaining experimental work, along with all data analyses, data presentation and interpretation as well as thesis writing was conducted by Andrew Madigan.

4.1 Abstract

Pathogenic fungi cause widespread destruction of crops and ecologically-important plants worldwide. To resist potential infections, plants have evolved a range of defences in response to pathogens, including the production of numerous secondary metabolites such as melatonin. Melatonin can act as an antioxidant or signalling molecule in plants, ameliorating the negative impacts of cellular ROS bursts induced by plants in response to exposure to abiotic and biotic stressors. However, melatonin is also synthesised in fungi, and to date very little is understood about the roles of melatonin in microbes. In this study, I investigated the *in vitro* growth responses of fungal pathogens: *Sclerotinia sclerotiorum*; *Botrytis cinerea* and *Fusarium oxysporum* f.sp. *vasinfectum* to two abiotic stresses [cold priming (4^{o}C for 2 days prior to treatments) and/or 2.5% v/v ethanol during treatments] under varying concentrations of melatonin (20 – 4000 µM). *S. sclerotiorum* was also inoculated onto seven-week-old tobacco (*Nicotiana tabacum*) leaves infiltrated with 200 µM melatonin. *In vitro* growth for all three fungi was significantly improved by melatonin (most notably at 1000 µM) under both abiotic stresses. Previous studies have demonstrated that melatonin may inhibit foliar pathogen infection when plants are treated with melatonin through the roots (Yin *et al.*, 2013; Lee *et al.*, 2014). In the current study, infiltration of tobacco leaves with 200 µM melatonin did not significantly affect the *in planta* growth of *S. sclerotiorum*. Overall, these findings suggest that fungal plant pathogens may utilise melatonin to cope with abiotic stresses, potentially to mitigate the impacts of plant defence strategies.

4.2 Introduction

Diseases caused by plant pathogens greatly impact food production throughout the world (Gonzalez-Fernandez and Jorrin-Novo, 2012; De Silva *et al.*, 2019). Cosmopolitan fungal pathogens such as *Sclerotinia sclerotiorum* (Lib.) de Bary and *Botrytis cinerea* Persoon ex Fries, known as white mould and grey mould fungus respectively, are of particular importance due to their ability to infect a wide variety of commercially important vegetables and crops (Boland and Hall, 1994; Kabbage *et al.*, 2015; AbuQamar *et al.*, 2017). Similarly, vascular wilt disease attributed to *Fusarium oxysporum* Schlecht., is particularly damaging within agriculture, as this cosmopolitan soil-borne species complex is pathogenic to most crops cultivated worldwide (Ali *et al.*, 2016; Lecomte *et al.*, 2016). The interaction at the site of infection between these necrotrophic fungi and a host plant is a highly complex and sophisticated process. Degradative enzymes and various mycotoxins secreted by all three pathogens result in a burst of highly unstable reactive oxygen species (ROS) in the host (Fernández *et al.*, 1993; Williams *et al.*, 2011; Gonzalez-Fernandez and Jorrin-Novo, 2012; Mbengue *et al.*, 2016). These excess ROS can greatly impact essential plant host cellular activities as a result of damage to DNA, reduced enzyme activities, lipid peroxidation of cell membranes and protein denaturation (Tan *et al.*, 2012; Pietraforte and Malorni, 2014; Zhang and Zhang, 2014). This ultimately leads to host cell death via either programmed cell apoptosis or necrosis (Glazebrook, 2005; Horbach *et al.*, 2011; Pietraforte and Malorni, 2014).

Melatonin (N-acetly-5-methoxytryptamine) is a secondary metabolite produced ubiquitously in nature (Arnao and Hernandez-Ruiz, 2014; Hardeland, 2015). Melatonin can scavenge excess stress-induced ROS, by either acting as a highly efficient antioxidant (Hardeland, 1999; Reiter *et al.*, 2015; Reiter *et al.*, 2016), or acting as a signalling molecule, resulting, in part, in the upregulation/enhanced activities of ROS-scavenging enzymes (Rodriguez *et al.*, 2004; Weeda *et al.*, 2014; Zhang *et al.*, 2015). Numerous studies have reported the ameliorative effect of this indoleamine for plants upon exposure to various abiotic or biotic stresses (Hardeland, 2016; Chen *et al.*, 2018; Liang *et al.*, 2018; Sun *et al.*, 2018). Melatonin biosynthesis has been described in bacteria such as proteobacteria and cyanobacteria, as well as in various fungi including yeasts and filamentous basidiomycetes and ascomycetes (Manchester *et al.*, 1995; Hardeland, 1999; Muszyńska *et al.*, 2011; Muszyńska and Sułkowska-Ziaja, 2012; Tan

et al., 2014), however very little is known about the roles of melatonin in microorganisms. The limited data existing suggests that melatonin may be utilised by fungi to enhance tolerance towards abiotic stresses (Rodriguez-Naranjo *et al.,* 2012; Liu *et al.*, 2016), similar to the roles observed in plants towards abiotic stress resistance. Interactions between plant and pathogen induce a rapid oxidative burst at the early stages of infection, resulting in increased endogenous melatonin levels (Arnao and Hernández-Ruiz, 2015). Melatonin has also been found to improve resistance for plants against pathogen attack. For example, root pre-treatment of apple [*Malus prunifolia* (Willd.) Borkh. cv. Donghongguo] with 100 µM melatonin resulted in enhanced immunity to the foliar fungal pathogen, *Diplocarpon mali*. (Yin *et al.,* 2013). Exogenous melatonin enhanced expression of pathogenesis related (PR) genes including genes associated with salicylic acid biosynthesis in both *Arabidopsis* and tobacco leaves exposed to a bacterial pathogen, *Pseudomonas syringae* DC3000 (Lee *et al.,* 2014). Salicylic acid (SA) is a phytohormone, acting as an endogenous defence signalling molecule associated with pattern triggered immunity (PTI) and effector-triggered immunity (ETI) responses in plants (Delaney *et al.,* 1994; Vlot *et al.,* 2009). Endogenous levels of melatonin in *Arabidopsis* leaves infected with *Pseudomonas syringe* pv. Tomato (Pst) DC3000, were significantly increased only 1 hr post infection with resistance to infection also increased upon the pre-treatment with 20 µM melatonin, suggesting a key role in acute immune responses to pathogenic attack (Shi *et al.,* 2015a). Importantly, the mode of action of melatonin in relation to the enhanced plant resistance has not been elucidated as it is unknown whether melatonin acts to prime host resistance or impacts directly on the fungal pathogens.

In this article, *in vitro* growth responses of three pathogenic fungi (*S. sclerotiorum*, *B. cinerea* and *F. oxysporum* f. sp. *vasinfectum*) towards a highly effective ROS-producing stress, ethanol at 2.5% v/v (Asiimwe *et al.,* 2012; Liang *et al.,* 2016), following cold priming (4^{o}C for two days) were investigated (Baraldi *et al.,* 2002; Li *et al.,* 2015). Cold priming may reduce the tolerance of fungi to ethanol stress due to the enhanced accumulation of deleterious ROS (Tosi *et al.,* 2010; Miteva-Staleva *et al.,* 2015). A range of increasing concentrations of melatonin were applied to decipher how fungi can be responsive to this efficient ROS-scavenging molecule, therefore revealing the plasticity of fungi to stresses due to melatonin. Additionally, *in planta* growth of *S. sclerotiorum* was assessed on *Nicotiana tabacum* leaves infiltrated with a selected concentration of melatonin. The plant was challenged with the fungus with

and without infiltrated melatonin, in order to demonstrate whether plant ROS scavenging mediated by melatonin could alter the pathogen development. It was hypothesised that: 1) *in vitro* fungal growth under abiotic stress conditions is improved by exogenous melatonin; 2) the availability of melatonin at the site of infection alters the infection capability of *S. sclerotiorum*. This is the first report indicating that melatonin can benefit fungal plant pathogens by enhancing *in vitro* tolerance to abiotic stresses.

4.3 Materials & methods

4.3.1 Background and identification *of* fungal pathogens

An Australian strain of *S. sclerotiorum* UQ1280 (Sexton *et al.*, 2006) (originally isolated by researchers at University of Queensland, Australia), was obtained from Melbourne University in 2005 and stocks maintained by regular collection of sclerotia from passaged infection of various plant tissues. An Australian isolate of *F. oxysporum* f.sp *vasinfectum* (*Fov*) (Australian vegetative compatibility group 01111) was collected from infected cotton (*Gossypium* sp.) by Department of Primary Industries, Queensland, Australia (Gaspar *et al.*, 2014). A MAT1-2 strain of *B. cinerea* (DAR 51217) was originally collected from fruit rot on *Vitus vinifera* in Coonawarra, South Australia, Australia in May 2002, and maintained as single spore stocks on PDA slopes stored at 4^{o}C. The identity of all three pathogens was confirmed to species level by sequencing the ITS region by Australian Genomics Research Facility (AGRF).

4.3.2 *In vitro* growth of fungal pathogens

In vitro agar-containing inoculum was obtained by incubating *S. sclerotiorum*, *B. cinerea* and *Fov* in darkness at 22^{o}C in 150 mm Petri dishes containing Potato Dextrose Agar (PDA, (39 g/l), Oxoid, Adelaide, Australia). After 5 days, plugs (diameter = 7 mm) collected from the hyphal edge, were transferred to the center of 90 mm Petri dishes containing half strength (½) PDA (19.5 g/l), with the fungi mycellium side of the plug facing down, i.e. plate inoculation. Half strength media provided a longer *in vitro* growth period, and thus a more accurate indication of any growth effects due to melatonin under abiotic stress. The fungal growing media was supplemented, while still warm (i.e. ~55°C), with 1.5 ml of dissolved melatonin (Sigma Aldrich Pty. Ltd., NSW, Australia) to reach final concentrations per Petri dishes of 20, 200, 500, 1000, 2000 and 4000 µM melatonin. According to manufacturers instructions, melatonin requires

dissolution with an organic solvent at high concentrations (> 2 mM). All melatonin treatments thus contained a standardised amount of ethanol, thus resulting in a final concentration in the media of 2.5% v/v. A control treatment consisted of 2.5% v/v ethanol only in ½ PDA. All Petri dishes were sealed with parafilm and incubated horizontally at 22ºC in darkness .

The effect of an additional cold priming treatment to the fungi was investigated by incubating the inoculum-containing 150 mm Petri dishes to 4ºC in the dark for 2 days, prior to being transferred (i.e. plugs) onto melatonin/control Petri dishes, and incubated at 21ºC under the conditions as described above. The radial growth (mm) of *S. sclerotiorum*, *B. cinerea* and *Fov* colonies were determined 2, 4 and 9 days post inoculation (dpi) respectively by measuring the mean distance from the plug to the edge of hyphal growth at four points on the hyphal edge of the two perpendicular diameters. Each treatment was performed with a total of nine replicates, i.e. conducted in triplicate with each experiment composed of three technical replicates per fungus. Data were presented as means ± standard error. Nonparametric analysis of data by pairwise Wilcoxon rank sum tests in R determined statistical differences ($p < 0.05$) between treatments.

4.3.3 Infection assays of *S. sclerotiorum* on *N. tabacum* leaves +/- melatonin

Mature leaves of *Nicotiana tabacum* plants were infiltrated with melatonin and infected with *S. sclerotiorum* to investigate the effects of melatonin on the growth of a foliar pathogenic fungus at the site of infection. *S. sclerotiorum* was grown from a sectioned sclerotium on PDA in 150 mm Petri dishes in the dark at 22ºC, and agar plugs (7 mm in diameter) were cut from the edge of growth after 5 days. Melatonin was prepared to a concentration of 200 µM with a final solvent concentration of 0.05% v/v ethanol. Abaxial foliar infiltration of 7-week-old *N. tabacum* plants with 500 µl melatonin (200 µM) was conducted using 1 mL syringe. Infiltrated leaves were immediately inoculated with a *S. sclerotiorum* plug with mycelium side touching the leaf surface at the point of infiltration. Infiltrations with 500 µl 0.05% v/v ethanol were used as negative controls.

Infection severity was determined by the lesion area around the plug, with infected necrotic leaf tissue appearing light brown or white. Fungal growth measurements from eight leaves, with one inoculation per leaf, were used for the melatonin treatment. This consisted of four different plants, with two mature, fully expanded leaves per plant. Six leaves from three different plants were used for the control treatment. On two plants

(separate to melatonin or control infiltrations), a total of four leaves were mock inoculated using sterile PDA plugs (without fungi). Plants were incubated in a sealed and moistened plastic container within a glasshouse at 16^{o}C night-28^{o}C day for 72hrs. Treated leaves were photographed, and the lesion area of fungal growth measured for each infection using Image J^{TM} software (National Institutes of Health, Bethesda, MD, USA). Briefly, the area of infection was highlighted by adjusting the image brightness threshold and analysed by converting the saturated area to binary format (Abràmoff *et al.*, 2004; Sheffield, 2008).

4.4 Results

4.4.1 *In-vitro* growth responses of plant pathogenic fungi to melatonin under abiotic stresses

4.4.1.1 Negative growth effects by stressors

The *in vitro* growth of all fungi was negatively ($p < 0.05$) impacted by exposure to 2.5% v/v ethanol: mycelial growth decreased by 67.5%, 32.9% and 17.5% for *S. sclerotiorum* (Figure 4.1) , *B. cinerea* (Figure 4.2) and *Fov* (Figure 4.3) respectively. Stress due to cold priming (4^{o}C, in the dark for 2 days prior to treatments) alone had no negative impact ($p > 0.05$) on the growths of *Fov* or *B. cinerea*, while growth of *S. sclerotiorum* decreased by 26.2% and upon exposure to cold. The combination of both stresses resulted in an additional growth decrease by 22.0%, 12.1%, 0.3% for *S. sclerotiorum*, *B. cinerea* and *Fov* respectively compared to the effects due to ethanol alone (Figures 4.1 – 4.3). Therefore, overall exposure to both stressors resulted in growth decreases of 89.5%, 45.0% and 17.8% respectively compared to growth under unstressed conditions.

4.4.1.2 Effects of melatonin under abiotic stress conditions

The availability of melatonin resulted in an increase ($p < 0.05$) of *in vitro* growth for all three fungi when exposed to abiotic stresses (Figures 4.1 – 4.5). Across the range of treatments (20 – 4000 µM), melatonin had varying ameliorative growth effects for each fungus. In general, 1000 – 2000 µM melatonin resulted in the greatest ameliorative effects under abiotic stress for all three pathogens. In contrast, 20 µM resulted in the weakest ameliorative effect under the same conditions. Upon exposure to 2.5% v/v ethanol alone, 1000 µM melatonin resulted in relative growth recoveries of 40.4%,

33.5% and 51.8% for *S. sclerotiorum*, *B. cinerea* and *Fov* respectively. When exposed to two abiotic stressors, growth recoveries of 37.1% and 56.7% were observed at 1000 µM melatonin for *B. cinerea* and *Fov* respectively. After 8 days, the radial growth of *S. sclerotiorum* relative to the ethanol control for 1000, 2000 and 4000 µM melatonin treatments showed increases of 37.1%, 56.5% and 75.0% respectively upon exposure to cold and ethanol stresses combined (Figure 4.4). The radial growth of *Fov* exposed to both abiotic stressors

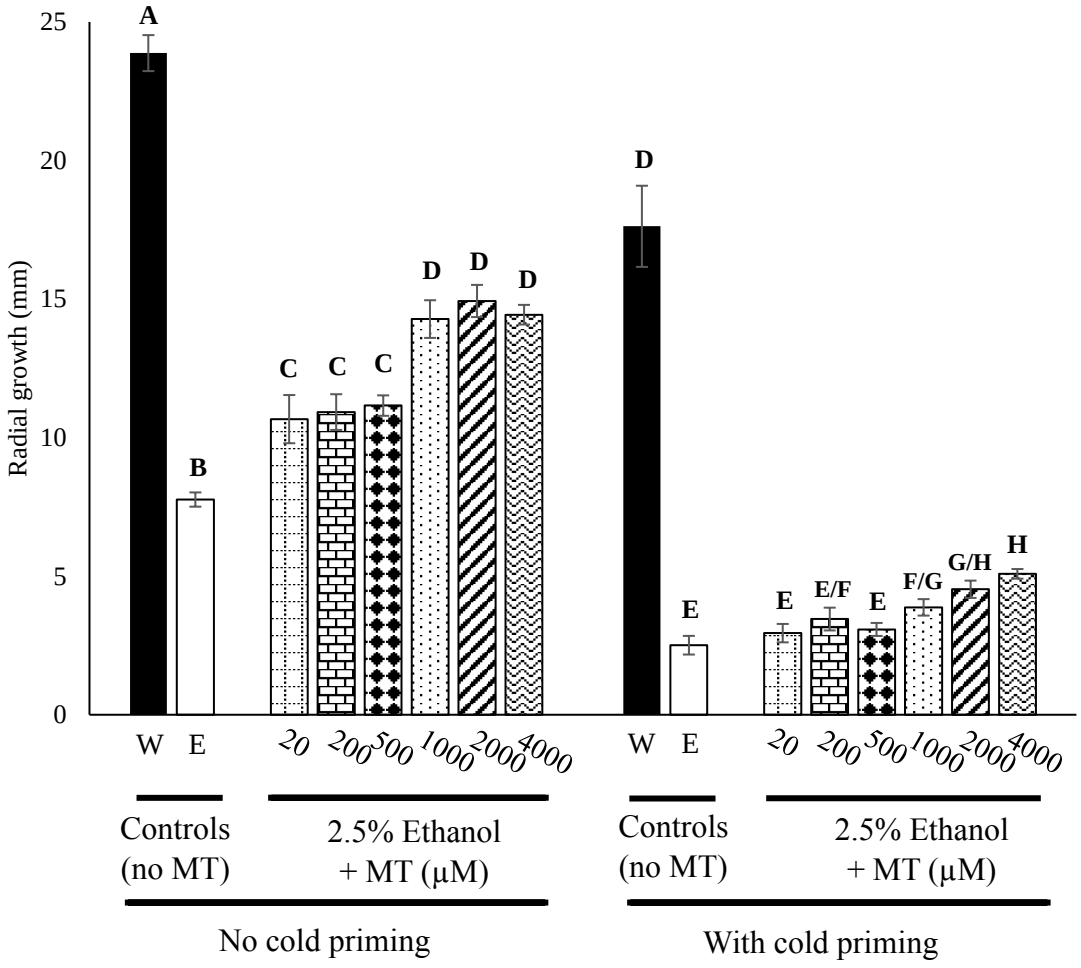

Figure 4.1: Radial growth responses of *S. sclerotiorum,* to treatments of melatonin (each with a standardised final concentration ethanol = 2.5% v/v) in ½ strength PDA (22ºC, darkness) after 2 days (Mean ± SE). Prior to treatments, *S. sclerotiorum* was initially grown in 150 mm Petri dishes containing PDA at 21ºC +/- 0.5ºC for 5 days. Cold priming represents exposure of *S. sclerotiorum* in 150 mm Petri dishes to a cold stress (4ºC) for an additional 2 days prior to transfer of hyphae to melatonin/control treatments. Nonparametric analysis of data by pairwise Wilcoxon rank sum tests in R determined statistical differences ($p < 0.05$ - represented by different letters).

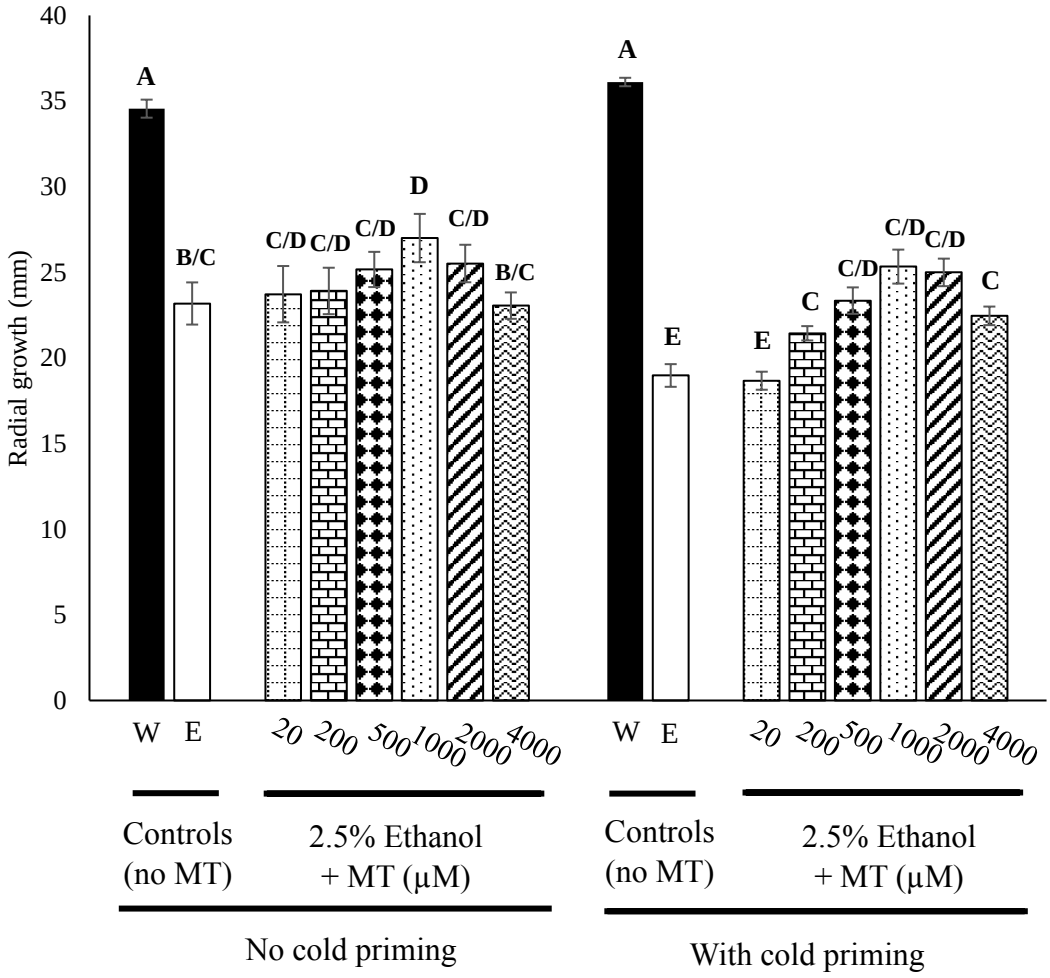

Figure 4.2: Radial growth responses of *B. cinerea* to treatments of melatonin (each with a standardised final concentration ethanol = 2.5% v/v) in ½ strength PDA (22ºC, darkness) after 4 days (Mean ± SE). Prior to treatments, *B. cinerea* was initially grown in 150 mm Petri dishes containing PDA at 21ºC +/- 0.5ºC for 7 days. Cold priming represents exposure of *B. cinerea* in 150 mm Petri dishes to a cold stress (4ºC) for an additional 2 days prior to transfer of hyphae to melatonin/control treatments. Nonparametric analysis of data by pairwise Wilcoxon rank sum tests in R determined statistical differences ($p < 0.05$ - represented by different letters).

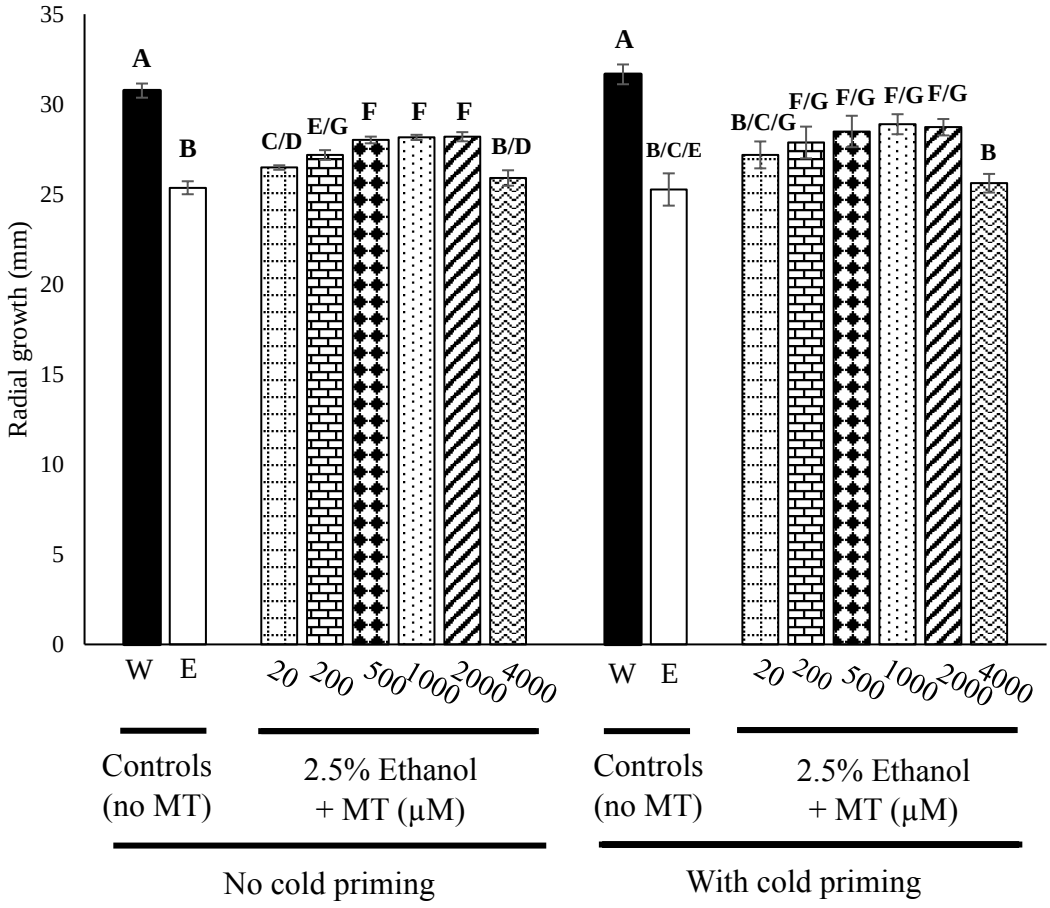

Figure 4.3: Radial growth responses of *F. oxysporum* f.sp. *vasinfectum* (*Fov*) to treatments of melatonin (each with a standardised final concentration ethanol = 2.5% v/v) in ½ strength PDA (22ºC, darkness) after 9 days (Mean ± SE). Prior to treatments, *Fov* was initially grown in 150 mm Petri dishes containing PDA at 21ºC +/- 0.5ºC for 12 days. Cold priming represents exposure of *Fov* in 150 mm Petri dishes to a cold stress (4ºC) for an additional 2 days prior to transfer of hyphae to melatonin/control treatments. Nonparametric analysis of data by pairwise Wilcoxon rank sum tests in R determined statistical differences ($p < 0.05$ - represented by different letters).

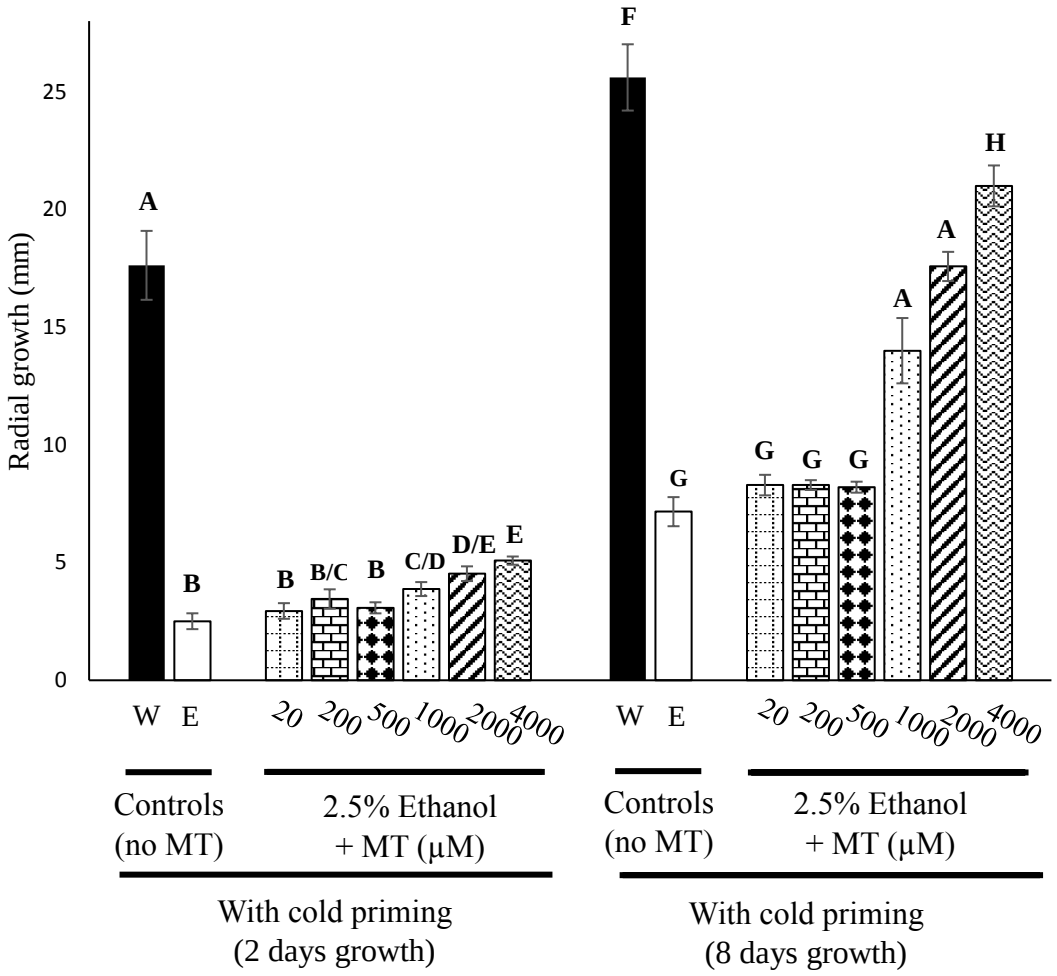

Figure 4.4: Radial growth responses of cold primed *S. sclerotiorum* to treatments of melatonin (each with a standardised final concentration ethanol = 2.5% v/v) in ½ strength PDA (22ºC, darkness) after 2 and 8 days (Mean ± SE). Prior to treatments, fungi were initially grown in 150 mm Petri dishes containing PDA at 21ºC +/- 0.5ºC for 5 days. Cold priming represents exposure of these fungi in 150 mm Petri dishes to a cold stress (4ºC) for an additional 2 days prior to transfer of hyphae to melatonin/control treatments. Nonparametric analysis of data by pairwise Wilcoxon rank sum tests in R determined statistical differences ($p < 0.05$ - represented by different letters).

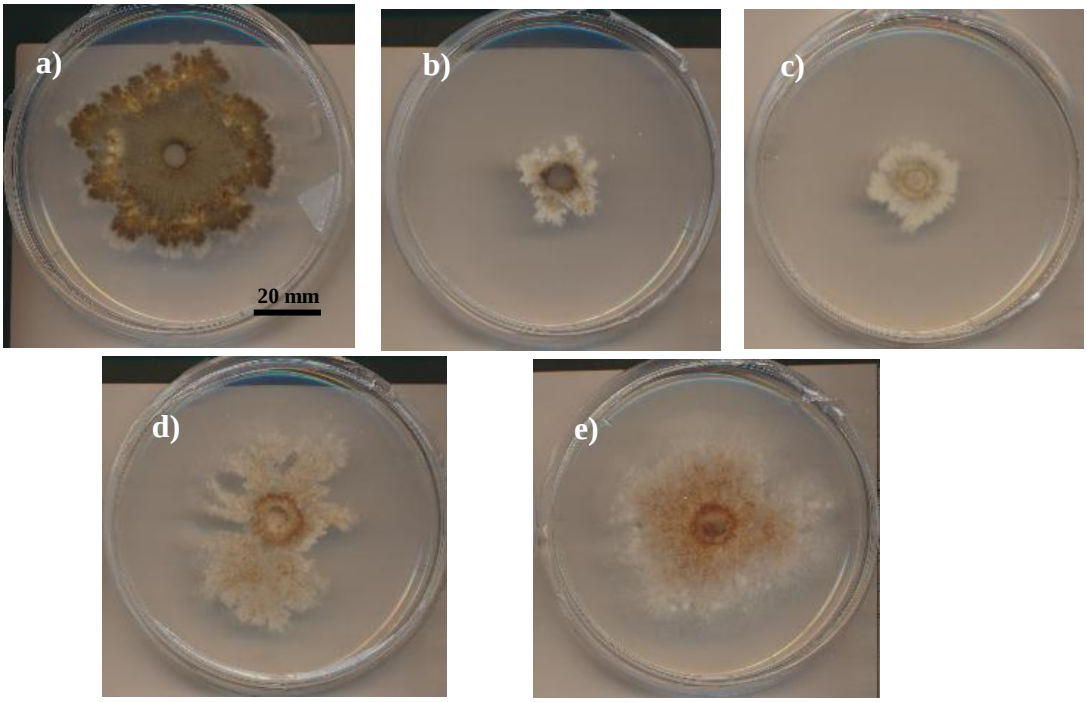

Figure 4.5: Radial growth of cold-primed *S. sclerotiorum* to treatments of melatonin (each with a standardised final concentration ethanol = 2.5% v/v) in ½ strength PDA (22ºC, darkness) after 12 days. a) Water only treatment ; b) Control (2.5% v/v ethanol); c) 20 µM; d) 1000 µM; e) 4000 µM melatonin. Cold priming consisted of initially growing the fungus in 150 mm Petri dishes containing PDA at 21ºC +/- 0.5ºC for 5 days, followed by exposure to a cold stress (4ºC) for an additional 2 days prior to transfer of hyphae to melatonin/control treatments.

4.4.2 *In planta* growth responses of *S. sclerotiorum* on *N. tabacum* leaves infiltrated with melatonin

Mature *N. tabacum* leaves were infiltrated with 200 µM melatonin and infected with *S. sclerotiorum* to investigate whether melatonin alters the infection capability of the pathogen. No significant differences were observed between the area of infections for melatonin-infiltrated leaves compared to controls (t-test; F = 0.805; p = 0.391) (Figure 4.6a).

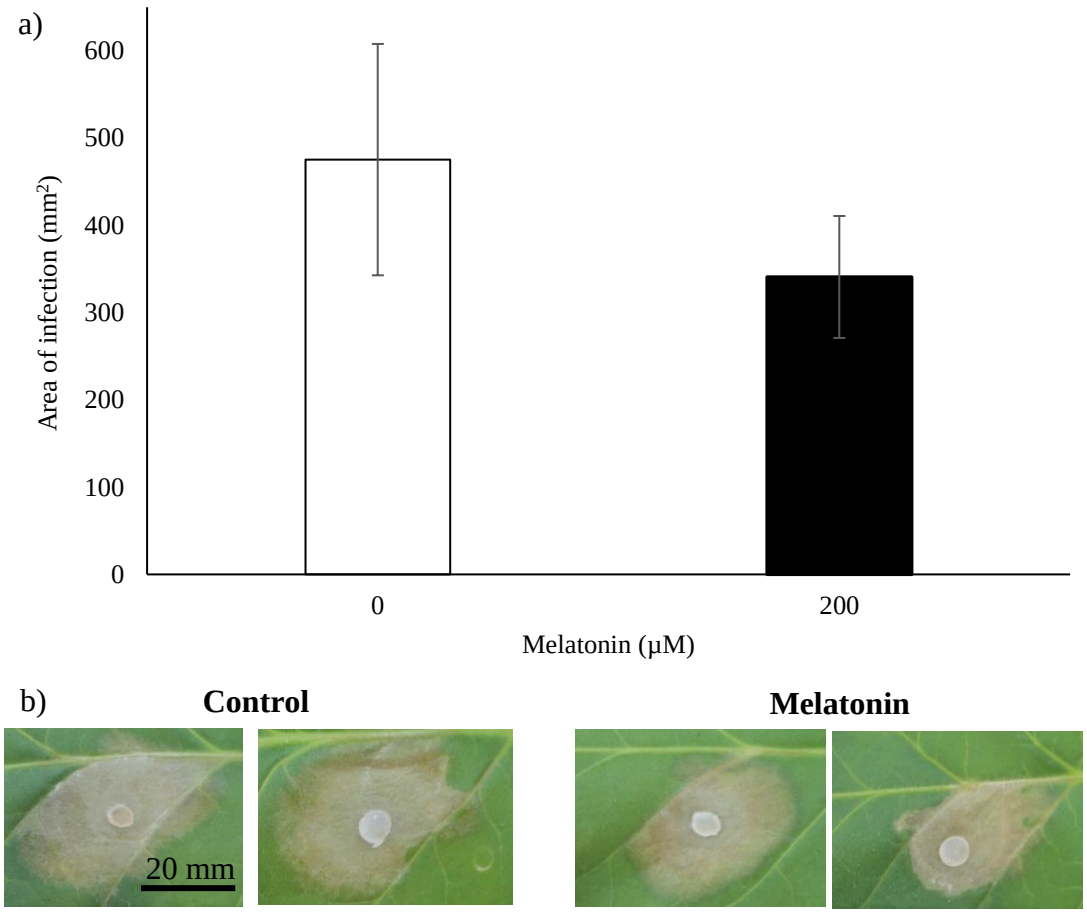

Figure 4.6: a) Growth of *S. sclerotiorum* on mature 7-week old *Nicotiana tabacum* leaves after 72 hours (Mean ± SE); b) leaf phenotype of fungal infection after 72 hrs. Leaf tissues were syringe-infiltrated with 500 µl of 200 µM melatonin (dissolved in 0.05% v/v ethanol) immediately prior to infection. Control (0 µM melatonin) treatments involved leaves infiltrated with 500 µl of 0.05% v/v ethanol. No significant differences ($p > 0.05$) were observed between the area of infections for melatonin-infiltrated leaves compared to controls.

4.5 Discussion

The present study investigated the *in vitro* growth responses of pathogenic filamentous fungi (*S. sclerotiorum*, *B. cinerea and F. oxysporum* f.sp. *vasinfectum (Fov)*) to melatonin under abiotic stress conditions. I hypothesised that melatonin would enhance tolerance to abiotic stress as observed in plant-melatonin studies (Zhang *et al.*, 2015). I also hypothesised that application of exogenous melatonin at the site of foliar infection between *S. sclerotiorum* and *N. tabacum* would not alter the infection capability of the fungus. The results in the current study showed that melatonin consistently ameliorated the negative growth effects of cold and/or ethanol stresses (Figures 4.1 - 4.4), suggesting that melatonin may be associated with abiotic stress tolerance in pathogenic fungi. The interaction between *S. sclerotiorum* and *N. tabacum* was also not altered at the site of infection by foliar infiltration with melatonin. As plants may also utilise melatonin to cope with abiotic and biotic stressors (Tan *et al.*, 2012), it is possible that the infiltrated melatonin was used by both the plant and the pathogen, therefore mitigating any potential beneficial role the secondary metabolite may have exhibited for the fungus.

Ethanol (Sakaki *et al.*, 2001; Asiimwe *et al.*, 2012) and cold (Godinho *et al.*, 2013; Duarte *et al.*, 2017; Yogabaanu *et al.*, 2017) stressors can affect fungal growth to varying degrees. In the current study, *Fov* was the least impacted fungus under ethanol stress, with mycelial growth reduction by 17.5%. This is similar to another study where *F. oxysporum* strain 11C demonstrated *in vitro* ethanol tolerance up to 4% v/v (Hennessy *et al.*, 2013). The sensitivity levels of *S. sclerotiorum* and *B. cinerea* were similar to those reported previously (Liang *et al.*, 2016). Cold stress can greatly impact the growth rate of *B. cinerea* (Baraldi *et al.*, 2002), as well as enhance the sensitivity of *S. sclerotiorum* to other abiotic stresses (Li *et al.*, 2015). In contrast, *Fusarium* spp. have been found to be relatively tolerant to cold (Hoefnagels and Linderman, 1999). In the current study, *in vitro* growth of *B. cinerea* and *Fov* were unaffected by cold stress alone, while *S. sclerotiorum* growth decreased by 26% upon exposure to this stressor (Figures 4.2 & 4.3). One explanation for the differences between these observations and Baraldi *et al.* 2002 for *B. cinerea* may be due to the differences in duration of cold exposure of *B. cinerea* between the studies.

Melatonin is a secondary metabolite highly conserved throughout all domains of life (Manchester *et al.*, 2015). However, to date, very little is known about the roles of

melatonin in microbes (Tan *et al.*, 2014; Liu *et al.*, 2016). In the current study, melatonin exhibited no antibiotic properties across a 20-4000 µM concentration range (Figures 4.1 – 4.4). Some investigations have reported antimicrobial properties for exogenously applied melatonin. Melatonin was shown to inhibit the *in vitro* growth of the human bacterial pathogens *Staphylococcus aureus, Pseudomonas aeruginosa* and *Acinetobacter baumannii* at concentrations between 130-530 µM (Tekbas *et al.*, 2008). Similarly, melatonin was found to inhibit the *in vitro* growth of the pathogenic yeast, *Candida albicans*, however at a much higher concentration (1300 µM) (Öztürk *et al.*, 2000). Exogenous melatonin has not been reported to have the same impact on pathogenic filamentous fungi as melatonin had no *in vitro* growth effects for *Physalospora piricola, B. cinerea or Mycosphaerella arachidicola* (Wang *et al.*, 2001). In the current study, *S. sclerotiorum, B. cinerea* and *F. oxysporum* were chosen due to their cosmopolitan pathogenic activities in agriculture (Kabbage *et al.*, 2015; Lecomte *et al.*, 2016; AbuQamar *et al.*, 2017). For all three pathogens, growth under cold and/or ethanol stress conditions was enhanced upon the availability of exogenous melatonin. This suggests that melatonin may play an important role in abiotic stress tolerance in these fungi. My findings are comparable to another study that indicated an important role of melatonin associated with abiotic stress tolerance in the biological control filamentous fungus *Trichoderma* spp. (Liu *et al.*, 2016). In particular, endogenous melatonin levels for *Trichoderma asperellum* increased up to three-fold upon exposure to various chemical stressors e.g. cadmium. In contrast, a preliminary study reported inhibitory effects at 4000 µM melatonin towards a plant pathogen, *Alternaria* spp. (Arnao and Hernández-Ruiz, 2015). A recent study also found that melatonin (500 – 5000 µM; standardised in 1% DMSO) exhibited no significant effects on the *in vitro* hyphal growth of *B. cinerea* or *F. oxysporum* (Zhang *et al.*, 2018). The differences observed in the current study and these investigations however may be due to exposure of my fungi to intense abiotic stressors (cold & 2.5% v/v ethanol) in the presence/absence of exogenous melatonin. It is possible that fungal pathogens may utilise exogenous melatonin differently and thus for some genera, such as *Alternaria*, melatonin at high concentrations (e.g. 4000 µM) may act as a stressor.

The ability of melatonin to ameliorate the negative impacts of cold and chemical stressors has already been well documented in plants (Reiter *et al.*, 2015; Zhang *et al.*, 2015). Growth suppression due to cold stress was significantly alleviated in melon (*Cucumis melo* L.) upon foliar spray treatment with 200 µM melatonin (Zhang *et al.*,

2017). Similarly, cold tolerance in tomato (*Solanum lycopersicum* L. cv. MicroTom) was enhanced upon pre-treatment with 100 µM melatonin (Ding *et al.*, 2017a). Alkaline stressed tomato (*Solanum lycopersicum* L.) and salt stressed maize (*Zea mays* L.) both showed increased photosynthetic capacities upon treatment with melatonin (Liu *et al.*, 2015; Jiang *et al.*, 2016), while endogenous melatonin levels in Lupin (*Lupinus albus* L.) roots increased 12-fold under exposure to zinc (Arnao and Hernández-Ruiz, 2013). The abiotic stress tolerance of the pathogenic fungi in the current study may have been due to the capability of melatonin to reduce cellular ROS levels. Ethanol-induced ROS negatively impacts various aspects of cell functioning including cell membrane fluidity, intracellular proteins configuration and activities of various glycolytic enzymes (Bailey *et al.*, 1999; Hu *et al.*, 2007; Ma and Liu, 2010). Similarly, cold stress conditions applied in the current study (4^{o}C for 48hr) impacts lipid membrane integrity, thus inducing severe oxidative damage due to the excessive accumulation of ROS (Ding *et al.*, 2017a; Ding *et al.*, 2017b). Melatonin is a highly efficient antioxidant, with the ability to scavenge up to 10 ROS per molecule (Hardeland, 1999; Reiter *et al.*, 2016). Melatonin has also been shown to act as a signalling molecule in plants, resulting in the upregulation or enhanced activities of ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) (Rodriguez *et al.*, 2004; Weeda *et al.*, 2014; Zhang *et al.*, 2015). As enhanced ROS cellular levels are a strongly associated with exposure to abiotic stress, the direct or indirect capability of melatonin to scavenge ROS may be a key role aspect of tolerance by fungal pathogens to abiotic stresses.

The current *in vitro* study demonstrated that melatonin assists abiotic stress tolerance in *S. sclerotiorum*, potentially due to reduction of stress-induced ROS. As plant defence responses exert a stress upon an infecting pathogen, I investigated whether melatonin infiltrated at the site of infection may be utilised by the fungus to cope with the impacts of plant defences. Melatonin did not significantly alter the degree of necrotic infection by *S. sclerotiorum* towards *N. tabacum* (Figure 4.6, t-test; $F = 0.805$; $p = 0.391$). One explanation for this result may be due to both the fungus and host utilising infiltrated melatonin to cope with the stress responses of infection. It is possible that the infection pressure by *S. sclerotiorum* may have been too high in the current study to observe biotic resistance by the host plant upon the availability of melatonin. Various other studies have demonstrated that melatonin enhances plant resistance to a biotic stressor. For example, endogenous melatonin levels increased significantly within 3 hours of

infection by *Pseudomonas syringae* pv. Tomato (Pst) DC3000 in *Arabidopsis*, indicating that melatonin is an important rapid response mechanism for biotic stress in plants (Shi *et al.*, 2015b). In addition, resistance of *Arabidopsis* to infection by Pst DC3000 was increased upon the pre-treatment with 20 µM melatonin (Shi *et al.*, 2015a). A recent study found that exogenous melatonin (200 mg/L) reduced the lesion length of rice bacterial leaf streak (BLS) disease, caused by *Xanthomonas oryzae* pv. *oryzicola* (Xoc) by up to 23% on rice strains susceptible to the bacteria. Similarly, foliar pre-treatment with melatonin significantly reduced infection capability and reduced the incidence of BLS by 17% (Chen *et al.*, 2019).

4.6 Conclusion

The results of this study demonstrated that exogenous melatonin enhanced tolerance to abiotic stresses for the three pathogenic filamentous fungi examined. To my knowledge, no previous study to date has demonstrated this association in fungal phytopathogens. Exogenous melatonin infiltrated at the site of infection did not alter the interaction between *S. sclerotiorum* and the host plant, possibly because the plant and fungus both utilised melatonin to cope with excess stress-induced ROS. Future studies may explore potential anti-oxidative roles of melatonin in fungal pathogens by measuring endogenous levels of melatonin and ROS under normal and abiotic stress conditions. Further research is also required to determine if melatonin may be utilised by non-pathogenic fungi such as mycorrhizae, endophytes (e.g. *Trichoderma* spp.), and saprobes to cope with abiotic stresses.

4.7 References

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Chapter 5 General Discussion

5.1 Overview

Over the past decades, increasing global demands on crop production and food quality have resulted in the development of unsustainable agricultural practices. For example, toxic chemicals such as various pesticides, have been applied to crops to kill insect herbivores and microbial pathogens that may otherwise have threatened the yield (Oerke, 2006; Murray *et al.*, 2013; Oliveira *et al.*, 2014; Deutsch *et al.*, 2018). However, pesticides can have acute and chronic health effects on humans exposed to the chemicals, and for those who consume the foods (Bonner and Alavanja, 2017; Bortoli and Coumoul, 2018; Cozma *et al.*, 2018). Concurrently, pesticide residues can have detrimental effects on the health and diversity of wildlife and the surrounding ecosystems (Costantini, 2015; Melchett, 2017; Budzinski and Couderchet, 2018). Coupled with these anthropogenic stresses, crop yield is being heavily impacted on a global scale due to increasing occurrences of extreme weather conditions such as droughts and heat waves caused by climate change (Ali *et al.*, 2017; Leng, 2017; Matiu *et al.*, 2017). The development of innovative agricultural practices is therefore an essential priority to ensure a healthier and more sustainable production of food throughout the 21st century (Morton and Abendroth, 2017; Shao *et al.*, 2019).

In recent years, research has been investigating a potential role of compounds naturally-produced by plants (e.g. secondary metabolites) as a means for enhancing tolerance to abiotic and biotics stresses (Khan *et al.*, 2008; Kang *et al.*, 2009; Park *et al.*, 2009; Khan *et al.*, 2013; Yadu *et al.*, 2017). In this regard, attention has been directed towards the indoleamine, melatonin. An extensive body of evidence indicates that melatonin enhances plant tolerance to several abiotic stressors including cold, heat, drought, salinity and heavy metal contamination in soil as well as to biotic stresses from bacterial and fungal pathogens (Arnao and Hernández-Ruiz, 2013; Arnao and Hernandez-Ruiz, 2014; Zhang *et al.*, 2015; Hardeland, 2016). This has led to an interest in the use of melatonin in agriculture to reduce negative physiological effects from environmental stresses that affect yield and crop quality. Soil microbial communities have an essential role in plant growth and development (Wall and Virginia, 1999; Yao *et al.*, 2000; Kirk *et al.*, 2004; Wahid *et al.*, 2016). Therefore, prior to a large-scale application of melatonin in agricultural practices, it is essential to understand how soil microbes

respond to melatonin and the effects these subsequent microbial changes may have for crops.

The research conducted across the three chapters in this thesis explored how soil microbes respond to melatonin, whether on an individual or community level, under abiotic stress conditions (cold, ethanol, cadmium or salt) or unstressed. In Chapter 2, microbial communities associated with three agricultural soils were treated with melatonin and a cadmium or salt stressor. This study demonstrated complex community responses to treatments, showing considerable differences across the three agricultural soils sampled. The results also clearly indicated that bacterial communities were more responsive to melatonin and stress treatments compared to fungi.

Based on our findings in Chapter 2, we followed up this investigation with a more in-depth exploration of individual bacterial taxa responding to melatonin in two of the agricultural soils sampled (Soil C and W) in Chapter 3. Prior to the commencement of this study (mid 2017), there was no knowledge regarding how bacterial communities, including beneficial soil microbes such as plant growth-promoting rhizobacteria (PGPR), respond to melatonin at a community level in agricultural soils. This data showed that the relative abundances of some PGPRs altered (increased/decreased; $p < 0.05$) in response to melatonin. Overall, responses of bacterial communities to melatonin were highly complex, and extensive research of different soil types under different conditions (abiotic stress and unstressed) is required to gain a deeper understanding of the overall bacteria responses to melatonin and the key factors that determine these outcomes. Changes to competitive soil dynamics or slight changes to important physiochemical parameters may provide possible explanations for these significant shifts, however these components were not analysed in the current investigation.

Following investigations into responses of soil microbes to melatonin at a community level, the investigation reported in Chapter 4 focused on individual responses of cosmopolitan plant pathogenic fungi (*Sclerotinia sclerotiorum, Botrytis cinerea and Fusarium oxysporum* f.sp. *vasinfectum*) to melatonin under two abiotic stressor conditions (ethanol and / or cold). *In vitro* growth of the pathogens increased ($p < 0.05$) with melatonin under abiotic stress conditions. Based on these findings, ecologically-important soil microbes are responding to melatonin, however the interactions between

melatonin and individual microbes is complex and requires further research. It was also found that that exogenous melatonin (200µM) infiltrated at the site of infection with *S. sclerotiorum* on tobacco leaves did not alter the radial growth of the fungus, suggesting that melatonin at this concentration did not significantly affect the interaction between the pathogen and host plant. Collectively, the investigations conducted in this thesis show that soil microbes respond to melatonin and can be utilised by some fungi to cope with abiotic stresses.

Melatonin has many important roles in humans, such as maintaining a balanced circadian rhythm, healthy blood pressure levels and a general sense of wellbeing (Baker and Kimpinski, 2018; Kun *et al.*, 2019). Melatonin is safe for human consumption and studies in human clinical trials have reported beneficial effects of administration with very few side effect (e.g. 15 mg melatonin daily for 10 days improved mood status (Castaño *et al.*, 2019); 0.5 mg melatonin daily for 4 weeks reduced sleep-related impairments (Sletten *et al.*, 2018)). Therefore, higher amounts of melatonin in foods due to exogenous application on crops and vegetables has a potential beneficial effect on human health, especially if it results in a reduced dependency on pesticide practices in agriculture. One current key drawback to the potential agricultural application of melatonin is the cost of production. Melatonin powder (> 98% purity) currently costs AU$146 per gram (Sigma Aldrich Pty. Ltd., Australia), which is not cost-effective for use in agriculture. However, a recent study reported the production of melatonin by yeast (*S. cerevisiae*) using a glucose-based approach (Germann *et al.*, 2016). This potentially offers a far more sustainable and economical method of producing melatonin. Further research is required to ensure the efficacy of the microbially-produced melatonin matches that of the pure powder form. Developing an affordable, large-scale method of producing melatonin is a vital logistical aspect regarding the future use of melatonin in agricultural practices.

5.2 Future studies

The community and *in vitro* analysis investigations conducted in this thesis showed that melatonin significantly affects soil microbes. Based on this finding, it can be concluded that future plant-melatonin research studies, involving application of melatonin through the soil should also take into consideration the effects of melatonin on the soil microbes as part of the investigations. Concurrently, any potential

applications of melatonin in future agricultural practices must also investigate the resulting shift to soil microbial communities to ensure that plant-soil ecology interactions are not negatively impinged in the long term.

Further research is also required to determine how soil characteristics (e.g. pH, content of nitrogen, organic C etc) influence the responses of microbial communities to melatonin, thus providing important information regarding agricultural soil parameters suitable for melatonin application. Further taxonomic research is also required to profile and isolate soil microbes responsive to melatonin under abiotic stress conditions. Microbes may be isolated by culturing in various media (e.g. agar and broths) supplemented with melatonin across a wide concentration range. The genes differentially expressed upon melatonin treatment in these microbes may subsequently be analysed by transcriptomics to explore potential functional associations between melatonin and abiotic stress tolerance in microbes, as reported in numerous plant-melatonin studies (Byeon *et al.*, 2015; Jiang *et al.*, 2016; Gu *et al.*, 2017).

Correspondingly, potential anti-oxidative or signalling roles of melatonin in bacteria or fungi may also be assessed *in vitro* by measuring endogenous levels of melatonin, ROS and ROS-scavenging enzyme activities under unstressed and abiotic stress conditions. Further *in vitro* studies could investigate how melatonin is utilised by fungal pathogens in comparison to non - pathogenic soil microbes such as mycorrhizae, endophytes plant growth promoting rhizobacteria and saprobes under various abiotic stresses (e.g. salinity, contamination, drought) and determine the conditions under which melatonin is optimally utilised.

Studies may also explore potential plant-microbe interactions in soil upon the availability of exogenous melatonin. This may include researching whether melatonin is secreted by plants in root exudate, as plants secrete other indoleamines (e.g. tryptophan and IAA) to manipulate a more beneficial microbial community structure in the rhizosphere (Narasimhan *et al.*, 2003; Badri and Vivanco, 2009; Etemadi *et al.*, 2014; Liu *et al.*, 2016). It is also important to explore if soil microbes secrete melatonin or if melatonin acts as a signalling molecule between soil microbes, similar to a functional role of the structurally comparable indoleamine, IAA (Fu *et al.*, 2015). Understanding the role of melatonin in soil microbial community dynamics may

provide vital information regarding the viability of melatonin application relating to future agricultural practices.

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